



Extraction of Polyphenol Oxidase Enzyme of Black Mulberry (*Morus nigra* L.)

Simge ÇOLAK¹ , Semih IŞIK² , Elif KALE BAKIR³ , Yonca YÜZÜGÜLLÜ KARAKUŞ^{4,*}

¹ Department of Biology, Kocaeli University, Kocaeli, Türkiye, **ORCID:** 0009-0009-5314-8993

² Department of Biology, Kocaeli University, Kocaeli, Türkiye, **ORCID:** 0000-0002-2035-6480

³ Department of Biology, Kocaeli University, Kocaeli, Türkiye, **ORCID:** 0000-0002-4266-9517

⁴ Department of Biology, Kocaeli University, Kocaeli, Türkiye, **ORCID:** 0000-0003-0286-8711

Abstract

This study focuses on developing a high-efficiency, cost-effective three-phase partitioning (TPP) purification strategy for polyphenol oxidase (PPO) from black mulberry (*Morus nigra*) fruit, known for its rich antioxidant profile. Reaction conditions were optimized, and maximum PPO activity was achieved in a system containing 100 mM sodium phosphate buffer (pH 7.0), 10 mM ascorbic acid, 0.5% (w/v) PEG 6000, and 50 mM K₂HPO₄. Under optimized TPP conditions, PPO was purified with an exceptionally high activity recovery of 177% and a 2.7-fold increase in purity, demonstrating the efficiency of this approach compared with conventional purification strategies. The methodological simplicity, scalability, and environmentally friendly nature of the process highlight its potential as a practical alternative for enzyme purification from natural sources. Overall, this study provides a cost-effective, efficient protocol for PPO recovery, offering significant industrial relevance for food biotechnology.

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1. Introduction

Oxidative stress is characterized by an imbalance between reactive oxygen species (ROS) generated during cellular metabolism and the antioxidants that neutralize them, resulting in ROS predominance¹. Disruption of this normally balanced system damages cellular structures such as lipids, enzymes, carbohydrates, proteins, and DNA. This damage can result in membrane disruption, molecular breakdown, lipid peroxidation, and ultimately cell death².

Oxidative stress is a fundamental cause of many diseases, including cancer, cardiovascular diseases, diabetes, and neurological disorders¹. ROS, the most common radicals in biological systems, are formed by the reduction of molecular oxygen through enzymatic reactions or the mitochondrial electron transport chain (ETS)¹. ROS, which are continuously produced by both metabolic processes such as cellular respiration and environmental factors such as UV radiation, smoking, and pollution, include both free radicals and non-radical

oxygenated molecules³. The critical balance between the effects of free radicals is maintained by redox regulation mechanisms. This regulation plays a vital role in protecting living organisms from the harmful effects of oxidative stress⁴.

Antioxidants are defense systems that prevent ROS formation or prevent cellular damage by scavenging existing free radicals^{5,6}. Their main functions are to remove excess radicals, protect cells, and prevent disease development⁷. They also extend the shelf life of products by preventing food oxidation. The biological effectiveness of antioxidants is determined by their chemical structure, structure/activity relationships, and natural origin⁸.

Polyphenol oxidases (PPOs), which are antioxidant enzymes, play a role in pigmentation, root formation, and environmental defense by oxidizing phenolic compounds. PPOs are classified into three groups: laccases, catechol oxidases, and tyrosinases; sometimes peroxidases are also included in this group⁹. Copper atoms are a fundamental requirement for all PPOs; however, laccases contain four copper atoms, while catechol oxidases and tyrosinases contain two¹⁰. Laccases are enzymes that can be obtained

* Corresponding Author: yonca.yuzugullu@kocaeli.edu.tr



from different organisms and are associated with lignin biosynthesis and stress response in plants¹¹. Tyrosinases, which are commonly found in nature, play a role in melanin synthesis and wound healing, while having wide applications in pharmaceutical synthesis and biosensors¹². Catechol oxidases, on the other hand, act only on *o*-diphenols, protecting plant tissues against pathogens¹³.

PPOs cause enzymatic browning in foods and plant tissue cultures because of the oxidation of natural phenolics to quinones, leading to a loss of quality¹⁴. Despite these negative effects, PPOs have high industrial value in areas such as wastewater treatment, biosensor production, and the treatment of various diseases. The use of these enzymes, which are in high demand in the global market, is limited due to their high production costs¹⁵.

Numerous studies have investigated PPO extraction from fruits. Common methods include salt precipitation, temperature-induced phase separation, and various chromatography techniques¹⁵⁻¹⁷. These methods are generally time-consuming, difficult to implement, and expensive. Additionally, because purification involves multiple steps, enzyme loss occurs during the process, reducing overall enzyme recovery¹⁸. In contrast, the three-phase partitioning (TPP) technique, developed in recent years and increasingly adopted, enables purification of enzymes from crude extracts in a single step with high yield¹⁵. TPP is considered the best technique for PPO purification due to its ease of use, environmental friendliness, cost-effectiveness, and lower solvent requirements compared to traditional chromatography methods¹⁹. However, despite being a modern technique for enzyme extraction, studies on fruit PPO purification using TPP remain quite limited²⁰.

Black mulberry, which belongs to the Moraceae family, is native to India and Japan and grows in temperate and subtropical regions²¹. Black mulberry, a purplish-black, oval-cylindrical, and tart fruit, is rich in anthocyanins, phenolic compounds, and potassium^{22,23}. Thanks to its flavonoids, it possesses many biological activities such as antioxidants, antimicrobial, and anti-cancer properties²⁴. Used in traditional medicine for its blood sugar-lowering, cough-suppressing, and mouth and tooth sore-treating properties, black mulberry also contains 3.30 ± 0.36 µg/g lycopene^{23,25}. It can be consumed fresh or dried and is also used in the production of various foods such as molasses, juice, and marmalade²⁶.

Several studies in the literature examine the PPO enzyme in blackberry fruit. Eydurán and Aziti²⁷ aimed to stabilize the enzyme using acetate buffer and polyethylene glycol (PEG), while Kutlu et al.²⁸ used high-speed homogenization with acidified water and methanol mixtures. Engmann et al.²⁹ focused on enzyme recovery with a complex system containing detergent (Triton X-

100) and high salt concentrations. Mustafa et al.³⁰ reported the effects of solvent-free physical methods, such as microwave-assisted hydrodiffusion, on biological activities. However, most of these studies leave the enzyme at the crude extract stage or require lengthy dialysis and costly chromatographic steps for purification. In addition, the dense pigment structure of black mulberries produces impurities that are difficult to separate from the enzyme using conventional methods.

To address the methodological limitations mentioned above, the TPP system, a low-cost method that provides high recovery during enzyme extraction, was used. For high-yield purification of the PPO enzyme with TPP, the ammonium sulfate concentration, crude extract to t-butanol ratio, and system pH were optimized.

2. Materials and Methods

The chemicals used in the experimental studies were generally purchased from Merck (Germany), Sigma (Germany), and Biolife (Turkey). Spectrophotometric measurements were performed using a temperature-controlled Cary60 UV-VIS (Agilent) spectrophotometer.

2.1. Obtaining Fruit Samples

Fruit samples were purchased from various public markets and supermarkets in Kocaeli and Bursa provinces. Commonly used parts of the fruit samples were washed and stored at -80 °C.

2.2. Preparation of Crude Fruit Extracts

200 grams of fresh fruit were weighed and homogenized by crushing with a blender. 15 grams of the pureed fruit were weighed and placed separately into Falcon tubes. 15 mL of 0.1 M sodium phosphate buffer (pH 7.0) was added to them. After this step, sodium phosphate buffer + ascorbic acid + PEG 6000 + K₂HPO₄ extraction was performed³¹.

2.3. Purification of Polyphenol Oxidase (PPO) Enzyme

The crude extract was first subjected to acetone precipitation, followed by TPP. For this, 20 mL of crude extract solution was incubated with cold acetone at a ratio of 1:1 and 1:4 for 1 hour and overnight at -20°C³². After incubation, precipitation was applied by centrifugation at 10000xg at 4°C for 20 minutes. Then, the resulting pellet was left to dry at room temperature (24°C) for 1 hour and overnight³³. The test pattern used for the acetone

precipitation process is shown in Figure 1. The dried samples (Samples 1-8) were vortexed for 1 minute after adding 12 mL of sodium phosphate buffer (0.1 M; pH 7.0).

The extract obtained from the prepared solutions was used in a TPP system. For this, ammonium sulfate ((NH₄)₂SO₄) was added to the samples at concentrations ranging from 20% to 70% (20%, 30%, 40%, 50%, 60%, and 70% w/v) and vortexed until completely dissolved. Then, t-butanol was added to this mixture at different crude enzyme extract:t-butanol ratios (1.0:1.0, 1.0:1.5, and 1.0:2.0 v/v). The resulting mixture was vortexed for another minute and then incubated at room temperature (24°C) for 60 minutes. After incubation, phase separation was achieved by centrifugation at 4500xg for 10 minutes, separating the three phases (upper phase: t-butanol-rich,

intermediate phase: protein-rich, lower phase: ammonium sulfate-rich)^{34,35}. After TPP, the resulting intermediate phases were dissolved in 2 mL of sodium phosphate buffer (0.1 M; pH 7.0), and polyphenol oxidase activity and protein content were determined in the intermediate and lower phases (salt medium). The upper phase containing t-butanol, where no protein was expected, was removed from the medium using a pipette. Optimization of parameters (ammonium sulfate concentration, crude extract:t-butanol ratio, system pH) that selectively play a role in the separation of biomolecules in TPP was also performed. Crude extract activity was assumed to be 100%. For control, 24 “blind” systems consisting of ammonium sulfate, distilled water, and t-butanol (without crude extract) were used.

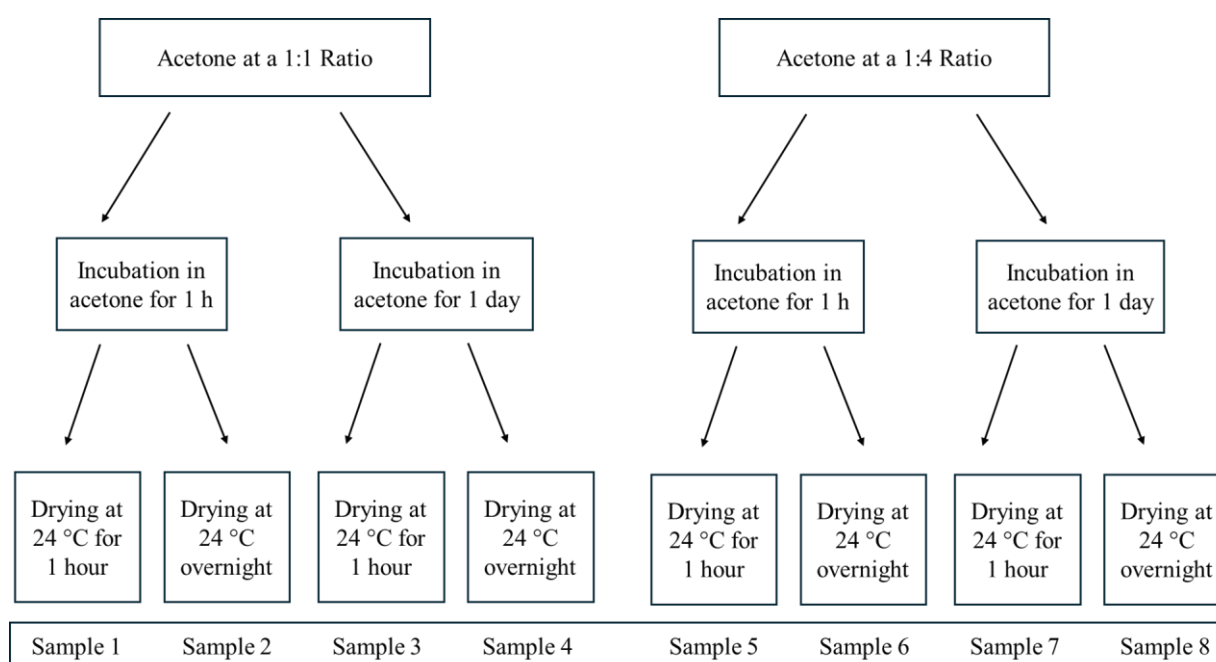


Figure 1. Test patterns used during the acetone precipitation process.

2.4. Determination of Polyphenol Oxidase (PPO) Activity and Protein Quantity

The presence of the PPO enzyme was detected by measuring the increase in absorbance at 420 nm (24 °C) with a spectrophotometer (Cary 60, Agilent). To assess enzyme activity, a sample cuvette was created by combining 200 µL of enzyme suspension with a substrate (catechol) solution in 2800 µL of sodium phosphate buffer (100 mM, pH 7.0). A control cuvette was similarly made, excluding the enzyme. The enzyme solution was prepared with a 100 mM sodium phosphate buffer (pH 7.0)³⁶. The amount of enzyme needed to produce 1 µmol of quinone per minute at room temperature is called a Unit of enzyme. The Bradford assay was employed to quantify the protein content of the samples³⁷. Bovine serum albumin (BSA)

was utilized to construct the standard protein graph, and the protein concentration of the samples was quantified spectrophotometrically at 595 nm.

3. Results and Discussion

3.1. Extraction of Mulberry Polyphenol Oxidase

In our previous study, the highest PPO activity (3753 U/mL) was measured from black mulberry fruit in a sodium phosphate buffer + ascorbic acid + PEG 6000 + K₂HPO₄ extraction medium³¹. In a study conducted with white mulberry (*Morus alba*)¹⁶, PPO extraction was performed similarly using 0.1 M sodium phosphate buffer (pH 6.5), 5% PEG and 10 mM ascorbic acid. In the

extraction performed with Iğdır apricot (*Prunus armeniaca* L.)³⁸, 0.5 M sodium phosphate buffer (pH 7.3) and 0.5% PEG were used and an enzyme activity of 567 U/mL was found. Since the highest PPO activity value was measured with this extraction from mulberry fruit, the crude enzyme solution prepared using this method was used in subsequent PPO purification trials.

3.2. Purification of Polyphenol Oxidase Enzyme from Black Mulberry

After activity measurements and protein quantity analyses were performed on 8 different samples, purification parameters were determined for each sample. According to Table 1, it was observed that the enzyme was

not purified in Samples 1, 2, 5, and 6. Among the other samples where the purification coefficient was calculated to be above 1, Samples 3, 4, and 8 showed activity recovery values above 100%. Since the purification coefficients were very close to each other in Samples 3 and 4, and the activity recovery value was determined to be higher in Sample 3 (124%), Sample 3 was used in the next purification step. In purification using organic solvents such as acetone, membrane-bound enzymes dissolve. Changes in solvent percentage are also used to separate different types of proteins¹⁵. In the literature, a 20-fold increase in activity has been observed in peach fruit purification using acetone precipitation³⁹.

Table 1. Acetone precipitation optimization

Sample Number	Specific activity (U/mg)	Total activity (U)	Total protein (mg)	Purification fold	% Activity recovery
1	869±43	6333±317	7±0.4	0.4±0.02	51±3
2	652±33	680±34	1±0.05	0.3±0.02	5±0.3
3	2399±120	15423±771	6±0.3	1.1±0.06	124±6
4	1492±75	14457±723	10±0.5	1.3±0.07	117±5
5	2902±145	11590±580	4±0.2	0.7±0.04	93±5
6	938±2	6342±317	7±0.4	0.4±0.02	51±3
7	2225±111	20491±1025	9±0.5	1.0±0.05	165±8
8	2589±129	9853±493	4±0.2	1.1±0.06	79±4

*The specific activity of the crude black mulberry extract is 2190 U/mg and the total protein content is 6 mg. Experiments were performed at room temperature and pH 7.0 in triplicate. Values are presented as mean ±SD.

3.2.1. Three-Phase Separation (TPP) Method

For the purification of the PPO enzyme, a three-phase separation (TPP) technique was used, which enables the rapid and efficient purification of biomolecules in a single step after acetone precipitation. Reasons why TPP is the best technique for PPO purification include the ease of use, environmental friendliness, economic efficiency of the system, and the fact that it requires less solvent than conventional chromatography techniques¹⁹.

The TPP process involves the selective distribution of target enzymes into one phase and the relegation of contaminating proteins to the other. A crude sample is treated with an inorganic salt, usually ammonium sulfate. The mixture is then amalgamated with an organic solvent, usually tert-butanol. The addition of t-butanol causes the mixture to stratify into three separate phases: a dense t-butanol phase at the top, an aqueous phase at the bottom, and a protein-containing interfacial precipitate¹⁹.

Although it is a modern technique for enzyme extraction, studies on fruit PPO purification via TPP are quite limited [20]. For high-yield purification of the PPO enzyme with TPP, the ammonium sulfate concentration, crude extract:t-butanol ratio, and system pH were also optimized.

3.2.1.1. Determination of Ammonium Sulfate (NH₄)₂SO₄ Concentration

Protein solubility is directly affected by the concentration of ammonium sulfate, making it one of the most crucial parameters in TPP systems. To maximize the recovery of the target protein, the concentration of (NH₄)₂SO₄ must be optimized since it promotes protein-protein interactions and precipitates proteins. Starting with a 20% (w/w) (NH₄)₂SO₄ concentration—as suggested in numerous papers¹⁸—experiments were conducted to evaluate concentrations up to 70% (w/w).

Figure 2 illustrates that elevating the (NH₄)₂SO₄ concentration to 60% (w/w) saturation enhanced the purification coefficient and activity recovery via the salting-out effect. The application of 60% (w/w) (NH₄)₂SO₄ yielded the highest purification coefficient of 2.7-fold and the greatest activity recovery of 177%. The observed reductions in purification characteristics at elevated ammonium sulfate concentrations may result from ongoing protein denaturation at increased salt levels⁴⁰. The optimal PPO purification coefficient and activity recovery were achieved with 60% (w/w) (NH₄)₂SO₄, hence this concentration was employed in further studies.

Examples of proteins purified with TPP at concentrations ranging from 0% to 80% (w/w) can be found in the literature. Some of these include plum (*Prunus domestica*; ⁴¹, avocado (*Persea americana*; ¹⁷, and Barbados cherry; ⁴².

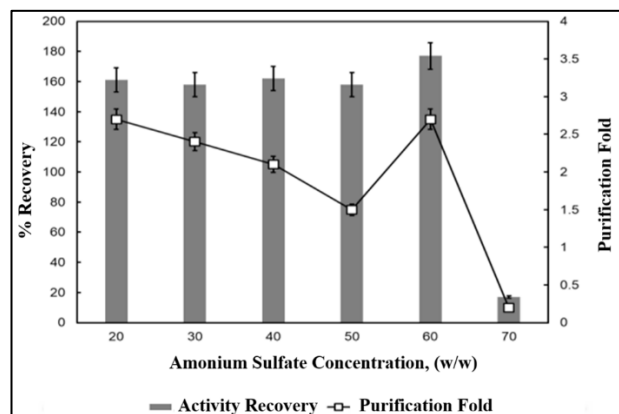


Figure 2. At different ammonium sulfate (w/w) concentrations, the results of the TPP system's intermediate phase were generated. Two milliliters of an enzyme extract crude with a total activity of 7506 units and three and a half milligrams of protein were used for the TPP assays. After subjecting the crude extract to ammonium sulfate concentrations ranging from 20 to 70% (w/w), 2 mL t-butanol was added. The experiments were carried out in triplicate at room temperature and a pH of 7.0. The data is shown as the average plus or minus the standard deviation (SD).

3.2.1.2. Determination of t-Butanol Ratio in Crude Enzyme Extract

Because it does not cause protein denaturation, t-Butanol is considered the best non-polar agent for TPP systems. While t-butanol and ammonium sulfate may not work together synergistically at low concentrations, the increased viscosity of the solution at high concentrations will certainly hinder intermolecular interactions. Therefore, it is necessary to modify the concentration of t-butanol added to the mixture to achieve efficient protein cleavage^{18,43}. In line with previous works, crude extract:t-butanol ratios varying from 1.0:1.0 to 1.0:2.0 (v/v) were evaluated with 60% (w/w) $(\text{NH}_4)_2\text{SO}_4$. Figure 3 shows that the best results were achieved with a 1:1 (v/v) ratio of crude extract to t-butanol. In contrast, extract:t-butanol ratios of 1.0:1.5 and 1.0:2.0 produced activity recoveries that were equivalent to one another. However, starting at 1.0:1.0, the purification coefficient showed a decreasing trend. A common belief is that the best ratio for obtaining TPP is 1:1 (v/v)¹⁵.

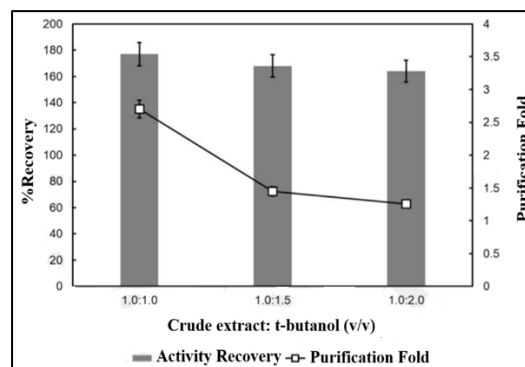


Figure 3. The TPP system's intermediate phase results were obtained at varying crude extract/t-butanol concentrations (v/v). The crude enzyme extract was saturated with 60% (w/w) $(\text{NH}_4)_2\text{SO}_4$, and then different volumes of t-butanol were stirred in. The experiments were performed three times with each set of conditions: room temperature, pH 7.0. The results are shown as the mean plus or minus squared.

3.2.1.3. Determination of pH of Triple-Phase Separation (TPP) System

The target protein's phase separation is also affected by the TPP system's pH. This is because electrostatic interactions between phases and proteins are impacted by variations in pH, which ionize amino acids in proteins. This could lead to a shift in the protein of interest's phase distribution. There is a direct correlation between the pI value of the target protein and its precipitation tendency in TPP systems, hence knowing this value is essential^{18,43}. This study examined the impact of pH on PPO distribution at pH values ranging from 4.0 to 8.0 in the presence of t-butanol and crude enzyme extract in equal quantities. The ammonium sulfate concentration remained constant at 60% w/w. The shaking time and temperature were likewise maintained at a constant level. Figure 4 shows that at pH 7.0, purification was 2.7 times higher and the recovery was 177% higher. Below pH 7.0, the purification coefficient is less than 1.0, meaning no purification occurs. At pH 8.0, the enzyme is more widely distributed in the intermediate phase, and purification is possible. However, the purification coefficient is lower than at pH 7.0. During TPP, an increase of over 100% was observed in enzyme activity for all parameters examined. Enzyme structural flexibility may account for this observed rise in enzyme activity⁴⁴. Numerous enzymes, including invertase, protease, amylase, peroxidase, laccase, catalase, pectinase, α -galactosidase, and trypsin inhibitor, exhibit this form of activation during TPP¹⁸. According to the findings of the study, a TPP system that contained 2 mL of crude enzyme extract that was saturated with 60% (w/w) $(\text{NH}_4)_2\text{SO}_4$ and 2 mL of t-butanol at a pH of 7.0 was able to purify black

mulberry PPO as much as 2.7 times while recovering 177% of its activity.

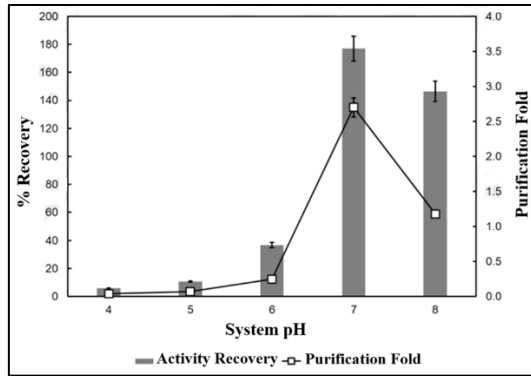


Figure 4. Findings from the TPP system's intermediate phase when manufactured at various pH levels. A solution with a pH between 4.0 and 8.0 was prepared by concentrating the crude enzyme extract with 60% (w/w) ammonium sulfate. Then t-butanol was added in the same volume. All trials were performed in triplicate at room temperature. The mean $\pm$ standard deviation is used to display the values.

Similar reports on the successful purification and characterization of many enzymes using TPP are available in the literature. In general, although the TPP system provides the desired proteins with lower purification yields than chromatographic methods, the activity recovery of the protein of interest from the system is consistently higher than that from other techniques. This feature, along with the other advantages mentioned above (ease and low cost of application, reduction of protein loss, etc.), makes TPP systems ideal for industrial enzymes¹⁸. However, there are only a limited number of reports in the literature on the purification of polyphenol oxidases using TPP. To date, only six examples of TPP and PPO purification have been reported^{9,35,40,45,46}, with only one involving fruit PPO²⁰. Using TPP, Niphadkar et al.⁴⁵ were able to achieve 6.3-fold purification and 70% activity recovery when they purified PPO from potato peelings. In another work, the PPO extracted from horseradish was purified by a factor of 3.59, and it recovered 68.75% of its activity after being subjected to two cycles of TPP³⁵. Recovering PPO in the lower aqueous phase, Saki et al.⁴⁶ achieved 8.4-fold purification using TPP from the edible mushroom *Lepiota procera*. Yuzugullu Karakus et al.⁴⁰ purified rosemary PPO 14-fold using TPP and achieved 230% activity recovery from the middle phase. In another study, fennel PPO was purified 20-fold with 120% activity recovery⁹. The only study on fruit PPO is on persimmon, where the enzyme was purified 12-fold with 150% yield using the TPP method. The present study is the first to use a similar method for PPO from blackberries. These findings indicate

that the TPP system can be successfully implemented for the purification of fruit-derived PPOs.

4. Conclusions

The PPO enzyme was successfully extracted and purified from black mulberry (*Morus nigra*), a fruit known for its high antioxidant content. The highest activity was obtained using extraction medium containing sodium phosphate buffer (100 mM, pH 7.0), ascorbic acid (10 mM), PEG 6000 (0.5%, w/v), and K₂HPO₄ (50 mM). Following extraction, acetone precipitation combined with the TPP method enabled efficient purification of the enzyme. Under optimized conditions, the PPO enzyme was purified 2.7-fold with an activity recovery of 177%. These results reveal that the TPP technique provides a rapid, economical and environmentally friendly alternative to chromatographic purification methods. The high recovery and simplicity of the TPP method highlights its potential for large-scale enzyme purification. Overall, the results suggest that black mulberry is a promising natural source of PPO and that the proposed purification strategy may be valuable for applications in food biotechnology and related industries.

Declaration of Ethical Standards

The authors of this article declare that the materials and methods used in this study do not require ethical committee permission and/or legal-special permission.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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