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Application of Aqueous Two-Phase System to the Purification of Persimmon Polyphenol Oxidase

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Abstract

Polyphenol oxidases (PPOs), which have recently become very popular among many researchers, catalyze the oxidation of phenolic compounds. Polyphenol oxidases are generally found in plants. Among them, persimmon (*Diospyros kaki* L.) is known as a good source for polyphenol oxidases. In this study, the polyphenol oxidase enzyme was purified from persimmon fruit using aqueous two-phase (ATPS). The optimized system was composed of 18% (w/w) PEG4000, 7% (w/w) NaH_2P0_4 and 1% (w/w) NaCl (pH 8.5, 25°C and 5 g). The PPO enzyme was obtained from the system by 4.8-fold purification with 191% activity recovery. The molecular weight of the enzyme, 28 kDa, was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In summary, it has been shown that the PPO enzyme can be obtained from persimmon in a short time and at low cost using a non-chromatographic method-ATPS.

Keywords: Polyphenol Oxidase; Aqueous Two-Phase System; Persimmon; *Diospyros kaki*

Abbreviations: PPO: Polyphenol Oxidase; ATPS: Aqueous Two-phase Systems; PEG: Polyethylene Glycol; PVPP: Polyvinylpolypyrrolidone; BSA: Bovine Serum Albumin.

Introduction

Polyphenol oxidase (PPO) is copper containing enzyme found in eukaryotic and prokaryotic organisms. PPO enzyme provides catalytic addition of oxygen (O_2) to phenolic substrates [1,2]. Due to their role in browning and pigmentation processes, PPO enzymes have been the subject of many inhibition studies. Additionally, their wide use in food, textile, and paper, pharmaceutical and cosmetic industries has increased the importance of PPOs even more [3-5]. The researchers have investigated a variety of PPO sources, including fruits and leaves from a variety of plants. This diversity in PPO enzymes results in varied extraction and purification methods applied by the researchers. These methods are chosen based on the enzyme's activity and purification fold, protein concentration, the presence of impurities, and the enzyme's final use. Because downstream processes account for a larger portion of an enzyme's production cost, having a thorough understanding of them for a specific enzyme is critical. For PPO extraction and purification, most research used a combination of salt precipitation, temperature induced phase separation, and chromatography. However, recent approaches such as three-phase partitioning and aqueous two-phase extraction have also been investigated [3].

Chromatographic methods are exceedingly expensive, difficult and time consuming, although they do provide a high level of purification. For chromatographic purification, a large amount of sample is needed. Additionally, the crude extract is often subjected to pre-treatment before purification such as ammonium sulphate or organic solvent precipitation. Equipment is also required to carry on the process [3]. The choice of the chromatographic method depends on the structure of the enzyme, its charge, its molecular weight, the amount, and impurity of the crude extract to be used. Although there are examples of single step used for PPO purification, most studies involved more than one chromatographic method together [6-10].

Aqueous two-phase systems (ATPS) based on phase partition-based biomolecule separation have received significant attention in the last decade due to their biocompatibility and high recovery efficiency with minimal aggregation and contamination. The ATPS process is carried out by using water-soluble polymers, salts-polymers, or salt-alcohol combinations [11,12]. ATPS is a polymer-polymer or polymer-salt system that forms two different phases of polymers with different hydrophobic properties (polyethylene glycol (PEG) and dextran) or one polymer one inorganic salt. PEG is generally preferred because it provides more advantage in terms of flammability, volatility, toxicity, and cost [8]. The target protein exhibits a selective distribution in two phases within the ATPS system that depend on the different physical and structural properties of the protein, the molecular weight of the polymer, the percentage of polymer salt, the system pH, the type, and number of neutral salts that can be added to the medium [13,14]. Therefore, all these parameters need to be optimized for the ATPS system. For PPO purification, it has been observed that most researchers have used a system containing a combination of PEG and a phosphate salt for [3].

Persimmon (*Diospyros kaki L.)* is a member of the Ebenaceae family. Known as persimmon in Turkey, this fruit is mostly grown in the North-East region of Turkey [15,16]. Persimmon fruits have been widely used in traditional Chinese medicine [17]. They contain many important compounds like calcium, potassium, and vitamin C, therefore having beneficial effects on health [16-19]. Additionally, they are very rich in phenolic content [16]. Considering persimmon as a good PPO source, we used their fruit to extract PPO enzyme using a non-chromatographic method, ATPS and investigated the efficiency of the ATPS method. The process parameters including PEG type and concentration, salt type and concentration, system pH and temperature were optimized.

Materials and Methods

Plant Material

Persimmon fruits (*Diospyros kaki* L.) were purchased from public market (Turkey).

Preparation of Crude Extract

Crude extracts were prepared according to the method reported Navarro, et al. [20] with some modifications. After washing the persimmon fruits with water, they were homogenized with a household blender. 15 mL of 0.2M phosphate buffer containing 0.1% (w/v) Polyvinylpolypyrrolidone (PVPP) and 0.1% (v/v) Triton X-100 were added to 15 grams of puree. The mixture is well mixed by vortex for 1 min. Then the homogenate was incubated at 20 min at +4 °C, centrifuged at 9000 x *g* and +4 °C for 30 min. Finally, the filtrate obtained from cellulose membrane was used as crude enzyme extract.

Protein Purification via Aqueous Two-Phase System

Different molecular weights of PEG (3000, 4000, 6000, 8000), various concentrations of PEG (5%, 14%, 16%, 18%, 20%) and 10% (w/w) of ammonium sulfate, magnesium sulfate, sodium sulfate, sodium bicarbonate, phosphate, and sodium chloride were prepared in distilled water. The system was set up in 15 mL centrifuge tubes by adding 5 gr crude extract with appropriate PEG and salt amounts. System parameters for the PEG/salt system have been determined according to the reports in the literature [21-24]. After ATPS, the purified protein was dialyzed against phosphate buffer at +4 ºC for 3 hours.

Enzyme Activity Assay and Protein Determination

To determine the PPO enzyme activity, the increase in the absorbance (420 nm) was measured using a spectrophotometer (Cary 60, Agilent) at 30 °C. To initiate the reaction, 0.1 ml of sample was added onto the substrate mixture (0.9 mL of 0.1M catechol in 0.2M sodium acetate buffer, pH5.5). The blank contained 0.9 mL substrate mixture and 0.1 mL of sodium acetate buffer. Protein concentration was measured according to the Bradford Method. Bovine serum albumin (BSA) was used as the protein in drawing the standard graph [25].

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE with 15% (w/v) gel was performed to check

the purity and determine the molecular weight of protein sample after ATPS [26]. The gel was stained according to the silver staining method [27].

Results and Discussion

Selection of PEG Type and Concentration for Phase Formation

It's been observed that most researchers have used a combination of PEG and a phosphate salt for PPO purification through ATPS method [28-30]. Therefore, optimization of PEG molecular weight is very important for efficient distribution of protein between phases. PEG acts on the protein phase distribution by changing the hydrophobic relationship between the protein and the polymer or other phase components. In the studies conducted so far, PEG molecular weight was used in the range of 4000-15000 and its concentration varied in the range of 5-18% (w/w) [3]. High-molecular weight PEG may inhibit the distribution of proteins to the upper phase while at the same time causing protein aggregation [31]. On the other hand, when low-molecular weight PEG is used, unwanted proteins as well as the target protein may interact hydrophobically with the polymer which would end up with unsuccessful separation [29,32]. In our study, the highest purification fold (3.4) and activity recovery (91%) were obtained from the bottom phase of the 18% (w/w) PEG4000/10% NaH_2PO_4 system, as shown in Table 1. Thus, this system was used for future experiments.

Phase composition*	PEG 3000		PEG 4000		PEG 6000		PEG 8000	
	PF	R (%)	PF	R (%)	PF	R (%)	PF	R (%)
PEG 5% + 10% NaH_2PO_4	0.3±0.1	11±1	1.4±0.3	34±1	0.3±0.3	4±0.7	0.3±0.2	6±0.7
PEG 14%+ 10% NaH_2PO_4	0.2±0.3	4±0.1	2.1±0.1	70±0.1	0.7±0.6	14±0.8	0.4±0.1	8±0.8
PEG 16% + 10% NaH_2PO_4	0.7±0.1	20±0.8	0.7±0.4	20±5	1.5±0.5	18±0.5	0.4±0.3	8±0.4
PEG 18% + 10% NaH_2PO_4	1.0±0.0	19±0.7	3.4±0.4	91±2	0.6±0.5	18±0.1	0.5±0.4	8±0.2
PEG 20%+ 10% NaH_2PO_4	5.9±0.2	10±0.2	3.1±0.1	79±1	0.4±0.03	11±1.0	0.6±0.3	8±0.4

PF: purification fold; **R:** activity recovery of bottom phase * 5 gr crude extract with catalase specific activity of 1033.6 U/mg protein and total protein of 1.58 mg in each tube, pH 8.0.

Table 1: Effect of PEG molecular weight and concentration on phase formation of persimmon PPO.

Selection of Salt Type and Concentration for Phase Formation

Selection of inorganic salt is another important parameter that affects the distribution of proteins. In general, phosphates are widely used for PPO extraction and purification. To examine the effect of salt type on the distribution of the PPO enzyme in the PEG / salt system, the purification fold and activity recovery values were obtained from the systems prepared using different salts at fixed concentration and compared for each other. As can be seen from the data in Table 2, the highest purification fold (3.4) and activity recovery (91%) were obtained from the system prepared using NaH_2PO_4. Thus, sodium phosphate was chosen for future experiments.

Phase composition*	PF	R (%)
PEG 4000 + 10% $(NH_4)_2SO_4$	1.6±0.0	35±0.7
PEG 4000 + 10% $MgSO_4$	2.1±0.3	50±2.0
PEG 4000+ 10% Na_2SO_4	1.6±0.5	16±4.0
PEG 4000+10% $Na_3C_6H_8O_7$	1.8±0.5	56±4.0
PEG 4000 +10% NaH_2PO_4	3.4±0.1	91±6.0

PF: purification fold; **R:** activity recovery of bottom phase * 5 gr crude extract with catalase specific activity of 1033.6 U/mg protein and total protein of 1.58 mg in each tube, pH 8.0.

Table 2: Effect of salt type on phase formation of persimmon PPO.

Salt concentration must also be optimized. Depending on the increase in the amount of salt, the hydrophobicity of the environment increases. Thus, the proteins are directed towards the polymer-rich phase. At high salt concentrations, proteins precipitate, which prevents the separation from being feasible [11,28,33-35]. Considering this, salt concentration varying from 5% (w/w) to 20% (w/w) was studied. Further concentrations resulted in salt precipitation in the bottom

phase and enzyme activity was completely lost. As shown in Table 3, the optimum conditions for PPO partition into the bottom phase were obtained from the ATPS prepared using 7% (w/w) NaH_2PO_4 with purification fold of 4.6 and activity recovery of 169%. The elimination of inhibitory proteins through the partitioning mechanism may explain the higher activity recovery of more than 100%. Increased flexibility in the enzyme structure may also result in improved catalytic activity. In liquid–liquid extraction, similar findings have been reported for enzyme extraction and purification [24].

Phase composition*	PF	R (%)
PEG 4000+5% NaH_2PO_4	1.9±0.1	120±8.0
PEG 4000+6% NaH_2PO_4	3.1±0.8	147±3.0
PEG 4000+7% NaH_2PO_4	4.6±0.9	169±4.0
PEG 4000+9% NaH_2PO_4	2.1±0.1	91±9.0
PEG 4000+ 10% NaH_2PO_4	3.4±0.7	93±9.0
PEG 4000+14% NaH_2PO_4	0.0±0.0	0.0±0.0
PEG 4000+16% NaH_2PO_4	0.0±0.0	0.0±0.0
PEG 4000+18% NaH_2PO_4	0.0±0.0	0.0±0.0
PEG 4000+20% NaH_2PO_4	0.0±0.0	0.0±0.0

PF: purification fold; **R:** activity recovery of bottom phase * 5 gr crude extract with catalase specific activity of 1033.6 U/mg protein and total protein of 1.58 mg in each tube, pH 8.0.
Table 3: Effect of NaH_2PO_4 concentration on phase formation of persimmon PPO.

Effect of Neutral Salt Concentration on Phase Formation

For a better distribution of proteins in ATPS, neutral salt with chaotropic effect can also be added to the environment. Because neutral salts cause an electrical potential difference between two phases, directing proteins to the more hydrophobic phase [36]. A variety of enzymes, such as amyloglycosidase [37] and formate dehydrogenase [38], have been selectively partitioned using the preferential response of proteins in PEG–salt systems in the presence of additional salt. NaCl is the most widely used neutral salt in ATPS. However, when added at concentrations above 7% (w/w), it's found that protein denaturation occurs [39]. Therefore, we have studied NaCl effect via adding this neutral salt at concentrations in the range of 0% to 7% (w/w). Adding a neutral salt (additive) to an ATPS usually changes the system's phase diagram as well as the properties of the partitioning solute [40]. In consistent with this knowledge, we observed a variation on purification parameters in response to change in NaCl concentration. The highest purification factor (4.8) and activity recovery (191%) values were measured at 1% (w/w) NaCl concentration (Table 4). Thus, the ATPS with 1% (w/w) NaCl was used for further purification of PPO.

Phase Composition*	PF	R (%)
PEG 4000+7% NaH_2PO_4+ 0% NaCl	4.6±0.9	169±40
PEG 4000 +7% NaH_2PO_4 +1% NaCl	4.8±0.1	191±30
PEG 4000+ 7% NaH_2PO_4+ 2% NaCl	1.5±0.5	92±10
PEG 4000+ 7% NaH_2PO_4+ 3% NaCl	1.3±0.4	84±15
PEG 4000+ 7% NaH_2PO_4+ 4% NaCl	1.0±0.1	54±2
PEG 4000 +7% NaH_2PO_4+ 5% NaCl	0.8±0.3	49±18
PEG 4000+ 7% NaH_2PO_4+ 6% NaCl	0.4±0.1	20±9
PEG 4000+ 7% NaH_2PO_4+ 7% NaCl	0.1±0.1	11±2

PF: purification fold; **R:** activity recovery of bottom phase * 5 gr crude extract with catalase specific activity of 1033.6 U/mg protein and total protein of 1.58 mg in each tube, pH 8.0.
Table 4: Effect of NaCl concentration for phase formation of persimmon PPO.

Effects of System pH on Phase Formation

The other important factor affecting the protein distribution in the ATPS is the system pH. If pH of the system changes, the net charge of protein of interest will also change. This will affect the interaction between target protein and the contaminants which will end up a change in the protein distribution between the two phases. As the pH rises, the proteins become negatively charged, and tend to accumulate in the top phase. The effect of pH on protein distribution is given in Table 5. Accordingly, the highest purification factor (4.8) and activity recovery (191%) values were obtained from the ATPS prepared at pH 8.0. Below pH 6.0 and above pH 8.5 the efficiency of the system decreased, while at pH 10.0 and 11.0 protein became denatured.

Phase Composition*	PF	R (%)
PEG 4000+7% NaH_2PO_4 + 1% NaCl at pH4.0	1.2±0.7	17±9
PEG 4000 +7% NaH_2PO_4+ 1% NaCl at pH5.0	1.7±0.2	43±5
PEG 4000+ 7% NaH_2PO_4+ 1% NaCl at pH6.0	2.1±0.1	88±3
PEG 4000+ 7% NaH_2PO_4+ 1% NaCl at pH7.0	3.7±0.6	154±1
PEG 4000+ 7% NaH_2PO_4+ 1% NaCl at pH8.0	4.8±0.1	191± 30
PEG 4000+7% NaH_2PO_4 + 1% NaCl at pH8.5	4.3±0.2	140±8
PEG 4000+7% NaH_2PO_4+ 1% NaCl at pH9.0	1.8±0.8	64±4
PEG 4000+ 7% NaH_2PO_4+ 1% NaCl at pH10.0	0.0±0.0	0±0
PEG 4000+ 7% NaH_2PO_4+ 1% NaCl at pH11.0	0.0±0.0	0±0

PF: purification fold; **R:** activity recovery of bottom phase * 5 gr crude extract with catalase specific activity of 1033.6 U/mg protein and total protein of 1.58 mg in each tube, pH 8.0.
Table 5: Effect of system pH on phase formation of persimmon PPO.

Molecular Weight Determination

For molecular weight determination, polyacrylamide gel electrophoresis was performed in the presence of denaturing SDS. The protein bands obtained at the end of electrophoresis are shown in Figure 1. Accordingly, an intense band around 28 kDa and some unwanted protein bands were observed on the gel. Studies that use plants as PPO source in the literature have shown that the molecular weight of PPO enzymes vary between 27 and 144 kDa. While PPO enzymes were observed as a single band on the gel in most of these studies, more than one band could also be seen in some of them due to the presence of different PPO isoforms [3]. The examples of PPO enzymes whose molecular weight has been reported include 26-36 kDa for potato PPPO [8], 51.3 kDa for broccoli PPO [41], 57.5 kDa for green bean PPO [42], 80.6 kDa for borage PPO [43] and 53, 112 and 144 kDa for mango PPO [44-46].

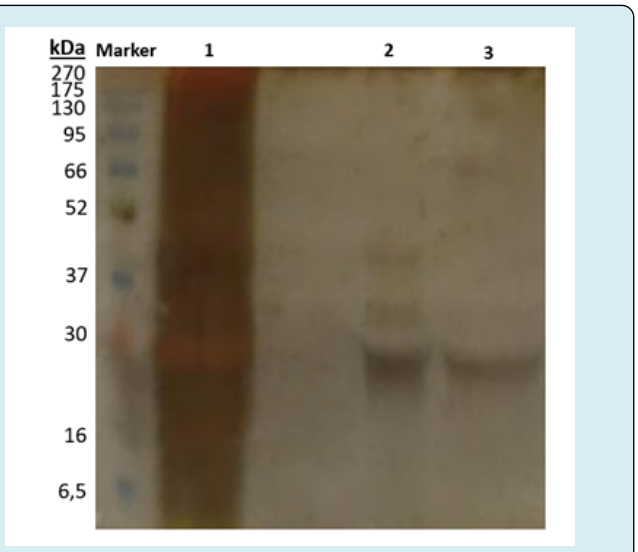

Figure 1: SDS-PAGE (15%) of PPO from persimmon purified with ATPS system. Lanes: Marker-molecular weight marker (Euroclone prestained protein sharpmass VII), 1-crude extract, 2-purified PPO collected from the bottom phase of ATPS, 3- purified PPO after dialysis for 3h at +4°C.

Conclusion

Fresh persimmon fruits were used as a PPO source and the enzyme was successfully extracted from crude enzyme extract using ATPS under optimized conditions. The system consisted of 18% (w/w) PEG4000, 7% (w/w) NaH_2PO_4, and 1% (w/w) NaCl (pH 8.0, 25°C). The purification factor and activity recovery values were determined as 4.8 and 191%, respectively. The molecular weight of the PPO was calculated as 28 kDa from SDS-PAGE. Overall results are highly promising for PPO extraction and purification in the first stage. The method for recovering PPO from persimmon is relatively inexpensive, simple, and effective.

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