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Extraction and Characterization of Polyphenol Oxidase From Plant Materials: A Review

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Abstract

Polyphenol oxidase (PPO) is a copper-containing enzyme that can be used for different applications including wastewater treatment and biosensing. Given these wide arrays of application, the reaction and biochemical characteristics of the enzyme must be known to further determine its other applications and to control the essential factors during processing. The purpose of this research was to review the different factors that influence effective extraction and characterization of PPO from plant materials. The pH of the extraction mixture, extraction temperature, type of buffer, mass to solvent ratio, extraction time, and additives are the factors that influence effective extraction of PPO from plant materials. Since PPOs taken from different plant sources have varied protein structures, these factors have different effects during extraction. The isolated PPO from the extraction process can be characterized based on its activity as a function of pH, temperature, and type of substrate, and on the values of its kinetic parameters (K_m and V_{max}). PPO isolated from different plant sources show varied optimum pH, optimum temperature, substrate affinity, and kinetic parameter values.

Keywords: Polyphenol Oxidase, Extraction, Characterization, Kinetic Parameters

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Introduction

Polyphenol oxidase (PPO) is a broad class of copper-containing enzymes responsible for the browning reaction observed in plants and animals.[1] The PPO found in humans is responsible for skin pigmentation including the development of freckles. In plants, PPO has been shown to be found inside the cell, particularly in the cell's plastids while phenolic compounds that act as PPO's substrate are found in the cell's vacuoles.[2,3] When the PPO and polyphenols are mixed either by senescence or because of injury, the reactions catalyzed by PPO commence. Both membrane-bound and soluble PPO are reported to be present in the thylakoid membrane and thylakoid lumen, respectively.[2,4]

There are three types of reactions that are catalysed by PPO: the hydroxylation of monophenols to form o-diphenols (also called as cresolase activity), the oxidation of o-diphenols to form o-quinones (also called catecholase activity), and the oxidation of p-diphenols to form p-quinones (also called laccase activity).[2,4,5] The last of the three reactions is particular to laccase, a sub-class of the broad PPO group, although laccase can also catalyse the oxidation of o-diphenols to o-quinones.[3] The three catalysed reactions, shown in Figure 1, utilize oxygen as an oxidant and co-substrate. Although cresolase activity is coupled with catecholase activity and may be viewed as a series of reactions, the catecholase activity is not always preceded by cresolase activity.[5] The o-quinones can then polymerize or form complexes with amino acids or proteins leading to the formation of brown pigments or polymers. In the food industry, the formation of these brown polymers is undesirable as it decreases the visual and nutritional quality of the plant leading to problems in its marketability.[6,7]

Different sources of PPO have been reported in literature including fungi, bacteria, seafood, and plants. PPO has been characterized from the mushrooms *Agaricus bisporus*, *Armillaria mellea*, *Lentinus edodes*, *Lepista nuda*, *Hypholoma fasciculare*, and *Pycnoporus sanguineus*.[8-10] PPO has also been produced intracellularly in the bacteria *Bacillus sp. A*.[11] The PPO found in shrimps and lobsters are responsible for the darkening of the crustaceans' parts during storage.[12-15] PPO has been extracted, purified, and characterized in various plants including sapodilla plum,[16] apple fruit,[17] avocado fruit,[18] marula fruit,[19] potato,[20] Chinese cabbage,[21] banana pulp,[22] cauliflower,[23] and truffles.[24] Different parts of a plant can also contain PPO. For instance, PPO has been shown to be present in different parts of the mango fruit including the pulp, peel, and sap.[25-27]

PPO has been reported to have several industrial uses. The ability of PPO to oxidize aromatic compounds has garnered significant interest. Previous studies have immobilized PPO

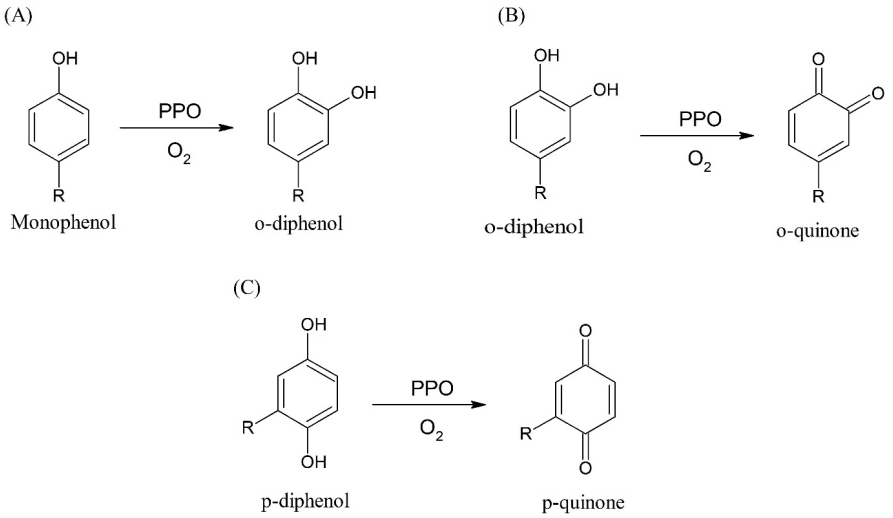

Figure 1. Three Types of Reactions Catalyzed by PPO: (A) hydroxylation of monophenols to form o-diphenols, (B) oxidation of o-diphenols to form o-quinones, (C) oxidation of p-diphenols to form p-quinones[2,4]; drawn using ACD/ChemSketch.

in different supports (e.g. membranes, gels, nanocomposites, nanoparticles) for the removal of phenols and other aromatic compounds from wastewater discharged from diverse industries like plastic, steel, paper, wood, etc.[28-31] Removal of these toxic compounds is important as they can cause health problems such as renal diseases, skin cancer, and even death.[32] The use of PPO in the bioremediation of wastewater is preferable since it uses molecular oxygen as the oxidant in its reactions. Other bioremediation methods using peroxidase or hemoglobin would need hydrogen peroxide, a costly chemical, in lieu of molecular oxygen.[32] Aside from its ability to treat wastewater, PPO immobilized in polymers, anionic clays, and carbon nanotubes was also used as biosensors to detect cyanide content and phenolic compounds in aqueous solutions.[33-35]

The presented studies highlight the importance of having an adequate supply of PPO. Hence, extraction and characterization of PPO from different plant sources is important. The aim of this research was to review the different factors that influence effective extraction of PPO from plant materials and its subsequent characterization. The isolated PPO from different plant materials was characterized and compared based on the activity as a function of pH, temperature, and type of substrate, and on the values of its kinetic parameters (K_m and V_{max}).

Extraction of Plant PPO

Since PPO in plants is shown to be an intracellular enzyme, it has to be removed through the disruption of the plant's cellular structure.[36] This involves breaking the integrity of the rigid cell wall housing the cellular fluid and organelles. After disruption of the cell wall, the cellular contents are released and should be solubilized in a solvent.[37]

In the entire extraction process, PPO's stability and its possible inactivation are prime concerns.[38] The development of the extraction procedure and the selection of extraction conditions should be geared towards the realization of these goals. A suitable buffer in ice-cold temperature is normally used in PPO extraction to solubilize the released enzyme while maintaining enzyme stability.[39,40]

Before the extraction of PPO, a few preparatory steps are done. The fresh plant tissue is chilled on ice, cleaned, and minced quickly. These steps should be done in as little time as possible to minimize endogenous degradation.[38] The mincing of the plant source into small pieces would improve the extraction efficiency by increasing the surface area exposed to the buffer.[38]

Compared to animal cells, bacteria, and yeasts, the disruption of plant cells is somehow challenging due to the presence of the rigid cell wall. To break the plant's cell wall, cellular disruption is usually done by vigorous mechanical methods.[40] Various equipment can be used to disrupt the plant cell's structure. These include ultrasonic sonicators, homogenizers (e.g. pestle and tube, Polytron), mortar and pestles, and blenders.[37,38] Among these, the laboratory blender is generally used for efficient homogenization of the plant tissue particularly for fleshy or nonfibrous tissues.[36,40] It uses shear forces to physically disrupt the cell wall and promote release of cell contents into the buffer. Care should be taken when mechanical disruption methods are used as they produce heat which must be controlled. Aside from the production of heat, foaming during blending should also be prevented to avoid oxidation and enzyme denaturation.[39]

The extraction and the corresponding assay conditions of PPO are sourced from different plant parts including the pulp, kernel, and seed are shown in Table 1. According to Table 1, there are different parameters that affect the extraction of PPO. These are (1) pH of the extraction mixture, (2) extraction temperature, (3) type of buffer, (4) mass to solvent ratio, (5) extraction time, and (6) additives used. It can be seen that different research studies used different parameter conditions in the extraction of PPO from different plant materials.

Factors Affecting PPO Extraction

pH of the Extraction Mixture

The pH of the extraction mixture, representing the

Table 1. Extraction Conditions for Polyphenol Oxidase

PPO Source	Extraction Conditions						Specific Activity (U/mg)	References
	Buffer	Mass:Solvent Ratio (g:mL)	Extraction Time	Additives	pH	Temp.		
Fruit pulp/meat								
Avocado	0.1 M sodium phosphate buffer	1:5	2 min	4 g PVPP, 0.5 mM PMSF	6.8	-	-	70
Manila mango	0.2 M sodium phosphate buffer	1:2	24 hrs	1% (w/v) PVPP, 1% (v/v) Triton X-100, 0.33 mg/100 mL aprotinin, and 0.1 mM PMSF	7	4°C	41	61
Banana	0.1 M sodium phosphate buffer	6:9	1 min	4% (w/v) Triton X-114	7.3	4°C	40.4	71
Blueberry	0.2 M phosphate buffer	1:1	30 min	4% PVPP, 1 M NaCl, 1% Triton X-100	6.5	-	-	72
Tainong mango	0.1 M sodium phosphate buffer	2:1	-	1% PVPP	-	-	-	52
Kernel								
Mango	0.01 M phosphate buffer	2:5	-	-	6	-	-	73
African mango	0.025 M phosphate buffer	1:10	3 min.	10 mM ascorbic acid	6.8	4°C	4.1 U/ml/mg	74
Pinhao	0.1 M sodium phosphate buffer	1:2	0.33 min	-	7	-	6.89	75
Chestnut	Acetone, 0.05 M sodium phosphate buffer	Acetone: 1:4	-	-	7	Acetone: -20°C	299	76
Seed								
Oil bean	0.025 M sodium phosphate buffer	-	-	-	7	-	7,670	77
Vanilla bean	0.15 M bis–tris-propane buffer	1:8	5 min	0.002 M EDTA, 0.003 M DTT, and 1.25 g PVPP	8	4°C	13,500	78
Field bean	0.1 M Tris-HCl buffer	1:5	16 hrs	2% (w/v) PVPP, and 1.2% (w/v) NaCl	7	4°C	2,013	68

For all pertinent studies except Sojo et al[71] one unit of PPO activity is defined as the change in absorbance of 0.001 per minute.
For Sojo et al[71] one unit of PPO activit is defined as the amount of enzyme that produced 1 µmol of the corresponding quinone/min.
Reagents: PVPP, polyvinylpolypyrrolidone; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NaCl, sodium chloride; PMSF, phenylmethylsulfonyl fluoride

concentration of H^+ ions, influences the optimal PPO activity and should be considered as one of the main factors in the PPO extraction.[41] Biochemical processes are highly sensitive to slight changes in the hydrogen ion concentration of the solution. In the isolation of plant enzymes, optimum results were found when the pH of the extracting medium is between 6.5 and 7.2.[42] It was also suggested that a buffer pH of around pH 7.0 should be used as the plant cytoplasm is around this value.[42] It is also good to note that the pH of the extraction buffer should be checked after putting the additives, if any, to the extraction buffer as these may change the pH of the buffers.[37] When the pH of the extraction buffer is greater than 7.5, the auto-oxidation of dihydroxyphenols to quinonoids and of protective thiol reagents is increased.[43] Many enzymes are active at narrow pH ranges, and exposure to pH values outside of these ranges may result to enzyme denaturation and irreversible inactivation.[44] Keeping the pH of the extraction buffer constant is also a factor that can help ensure the reproducibility of experimental results.[38]

The chosen buffer's pK_a should be close to the optimal pH of the enzyme as the buffering capacity of the buffer is maximum at the pK_a value and drops off quickly 1 pH unit from the pK_a. Generally, the enzyme's optimal pH should be within 1 pH unit from the buffer's pK_a value.[37] In addition, the buffer should not be used outside this range. The pH of the extraction buffer can be calculated using the Henderson-Hasselbalch equation in equation 1.

$$pH = pK_a + \log \frac{[\text{basic species}]}{[\text{acidic species}]} \quad (1)$$

Based on equation 1, the pK_a of the buffer is that pH where the concentrations of the basic and acidic species comprising the buffer are equal. It is important to note that this equation is only valid in the pH range of 3 to 11.[39] Outside this pH range, water's ionic species have to be included in the equation.

As seen in Table 1, all of the studies in the extraction of PPO from different plant materials utilize a pH value of 6.0-8.0. The effect of the extraction buffer's pH on the PPO activity has been studied. In the extraction of PPO from raspberry,

investigating and using a pH range of 5.0 to 8.0, the maximum PPO activity is recorded when the pH of the extraction buffer is set at pH 7.0.[45] In the extraction of PPO from banana roots, pH values of 5.5 and 9.0 were also investigated where the highest PPO activity is found for pH values of 7.0 and 9.0.[46] The authors surmised that the optimum pH of 9.0 was probably due to the phenolic substrate's auto-oxidation at this pH level.

Extraction Temperature

It is also important to control the temperature of the extraction mixture. Usually, extraction mixture temperatures are kept as low as possible (near 4°C) to prevent enzyme denaturation and minimize protease activity.[36,38] As seen in Table 1, the extraction temperatures are in the range of 0-4°C.

In the extraction of PPO in a laboratory scale, a blender may be used. When this equipment is used, it is recommended to use pre-cooled reagents and apparatus at the pre-determined temperature to prevent local elevation of temperature in the buffer during the blender operation.[36,39] Moreover, the blender is to be operated in short (15-30 seconds) bursts with adequate cooling time in between bursts.[36] To aid in keeping the extraction temperature as low as possible, the homogenization process is recommended to be performed in a cold room.[39]

Type of Buffer

Buffers consisting of an acid and its conjugate base (e.g. acetic acid and acetate) help maintain a stable pH when studying an enzyme.[37] Different types of buffers can be used in enzyme extraction. Each type of buffer is composed of different ions which can have varied effects on different enzymes.[39]

Several factors have to be considered in selecting an appropriate extraction buffer. These include the pH optimum of the target enzyme, specific effects of the buffer on the enzyme, subsequent purification methods, the buffer's sensitivity to temperature, its interaction with substrates and metals, and cost.[41] As mentioned, the buffer's pK_a should be close (at most 1 pH unit away) from the enzyme's optimum pH value. Following this criterion, a couple of buffers may be qualified to be used in extraction. In the extraction of 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyls, the use of potassium phosphate buffer produced over twice as much enzyme activity compared to when three other buffers (i.e. Bicine-KOH, Tricine-KOH, and 2-amino-2-ethyl-1,3-propanediol buffers) were used.[47] The effect of buffer type on the activity of *Candida rugosa* lipase has also been studied.[48] The authors investigated three buffer types: sodium citrate, sodium phosphate, and Tris-HCl. Results show that at 0.2 M equal concentrations, *C. rugosa* lipase has showed maximum activity in sodium phosphate buffer and least activity in Tris-HCl buffer. It can be concluded that the buffer ions interact with the amino acid residues of the enzyme's catalytic site and interfere with the catalytic mechanism resulting to different values of enzyme activity.[49]

Types of buffers also exhibit different characteristics which must be taken into consideration in buffer selection. Primary amine buffers are temperature-sensitive and are shown to interfere with the Bradford protein assay.[39] The pK_a of Tris buffer changes from 8.85 at 0°C to 8.06 at 25°C, so this should be considered when temperature is a concern. Tris-HCl buffers have also been reported to react covalently with proteins.[50] Some buffers also form complexes with metals present in the extraction medium. If this is a concern in the extraction process, the so-called Good buffers (e.g. HEPES) developed by Good et al[51] are preferred since they have low metal-binding capabilities compared to inorganic buffers. However, the Good buffers are more expensive than inorganic buffers. Some Good buffers also give false-positive colours when the Lowry protein assay is used.[39] Phosphate buffers, on the other hand, are usually used in enzyme extraction as they are compatible with most enzymes, do not cause enzyme inhibition or denaturation, and are also inexpensive.[39,50] As can be seen in Table 1, the majority of studies use phosphate buffer in PPO extraction. Its compatibility with PPO is evident based on the studies of Tsai et al[47] and Salis et al.[48]

Mass to Solvent Ratio

The ratio of the amount of plant material to extract PPO from the amount of extraction buffer is another variable which may be investigated. Better extraction is obtained when more buffer volume is used compared to the mass of plant source.[42] For every mass of plant source, it is recommended to use at least twice the buffer volume.[38] The mass to solvent ratio is a compromise between achieving maximum extraction and using minimum buffer volume. Inadequate buffer volume may result to change in pH during extraction which is an undesirable event as it may inhibit or possibly denature the enzyme, as discussed. The volume of the buffer should be chosen such that during blending, undue vortexing and sparging of air into the extraction mixture which can oxidize cellular compounds leading to enzyme inhibition is avoided.[43] In Table 1, all studies except the study of Wang et al[52] have used a minimum mass to solvent ratio of 1:2. The effect of changing the mass to solvent ratio during extraction has been studied. In the extraction of PPO from banana roots, changing the mass to solvent ratio from 1:10 to 1:20 increased the enzyme's activity from 115 U/g-s to 190 U/g-s.[46] The increase in the buffer volume possibly resulted to a better enzyme extraction. However, an increase in buffer volume does not always translate to higher enzyme activity. In the extraction of PPO from the apple fruit, when the ratio of the apple sample to buffer was changed from 1:5 to 1:2.5, the PPO activity increased by about 6-fold.[53] The authors posited that the increased ease in homogenization and in enzyme solubilisation may have led to the increase in activity.

Extraction Time

The extraction time is the amount of time used to disrupt the cell structure enabling the release of the PPO and other intracellular compounds. Concurrently, the extraction time is also the amount of contact and interaction times between PPO and these intracellular compounds which may cause

inactivation or denaturation of the enzyme.[40] It is for this reason that rapid extraction is important.[38] Keeping the extraction time as low as possible would also be economically preferable since lesser energy is used.

According to Table 1, the extraction time for PPO is also varied among different plant sources. The effect of extraction time on the PPO yield or activity has been reported to be different depending on the PPO source. A study on the extraction of wheat PPO indicated that extraction time has no major influence on the PPO activity.[54] Meanwhile, the enzyme activity significantly increased when extraction time increased for PPO extraction from potato peel.[55] These differences can be attributed to the differing properties of PPO when extracted from different plant sources.

Additives

As mentioned, upon disruption of the cellular plant structure to obtain the enzyme PPO, other intracellular compounds are also released which can mix with PPO and possibly cause its inactivation or denaturation. To remove these compounds or minimize their effects, different types of additives may be employed.[37]

Protease Inhibitors

One of the compounds released during cell disruption are proteases contained in the vacuoles of the plants.[2] Proteases, also called as proteinases, are proteolytic enzymes that cleave peptide bonds and break proteins down into individual amino acids.[37,38] It can also partially degrade the enzyme which can lead to poor conclusions about its size and structure.[41] The inactivation and removal of proteases is imperative in PPO extraction.[39,43] Some common protease inhibitors include PMSF (phenylmethylsulfonyl fluoride), benzamidine, aprotinin, EDTA, EGTA, pepstatin A, leupeptin, and antipain.[41]

Before using a protease inhibitor, it is important to understand its properties as some protease inhibitors can be toxic or unstable under certain conditions.[41] Cocktails of protease inhibitors are commercially available to use against two or more classes of proteases but care must be taken to ensure that it does not inhibit the activity of the enzyme.[38] The class of protease in an enzyme extract can be determined by using azocasein in the presence and absence of different protease inhibitors.[36]

In the early stage of enzyme extraction where the concentration of the target enzyme is low, the damage caused by proteases may be minimal. In addition, the presence of a wide range of proteins to which the protease act on also minimizes the action of the protease on the target enzyme.[41] The use of protease inhibitors is imperative when purification methods are adopted since the concentration of the enzyme in the buffer will increase.[36] A simple way to minimize the damaging effects of these proteolytic enzymes is to inhibit them by conducting the experiments at low temperatures such as 5-10°C.[5,36]

The presence of protease in mango has been reported. Ahmad et al[56] has reported the presence and extraction of protease in the horse mango (*Mangifera foetida* Lour) kernel. The protease obtained from this study has enzyme activity of up to 12.27 U/mL. However, the type of protease extracted has not been investigated. For the mango peel, the presence of serine proteases has been widely reported.[57-59]

Protective Agents

Plant cells may also contain endogenous phenolic compounds which interfere in extraction. These phenolic compounds are converted to polymeric pigments by PPO in the presence of oxygen.[44] These polymeric pigments can adsorb and inactivate the extracted enzymes.[42] To prevent this, it is recommended to add polyvinylpyrrolidone (PVP) or its insoluble counterpart polyvinylpolypyrrolidone (PVPP).[43] Both additives can adsorb the phenolic compounds by forming stable hydrogen bonds to the phenol groups preventing the formation of the polymeric pigments.[43,44] PVP and PVPP also do not interfere in enzyme assays, though PVPP has the added advantage of being easily removed in the clarification steps by either filtration or centrifugation.[36,40,42] Aside from PVP and PVPP, ascorbic acid may also be used as a reducing agent for the phenolic compounds converting them to phenolic substrates.[16] However, reducing agents have to be removed before the enzyme assay to prevent enzyme inhibition.[60]

As seen in Table 1, PVP and PVPP are commonly used additives in the extraction of PPO. Previous research has also studied the effect of these additives in the extraction of PPO. On the extraction of PPO from banana roots, the addition of 1% PVPP increased the PPO activity by almost 3-fold which indicates its effective role as a protective agent.[46] Cortez et al[16] studied the effect of PVP concentration on the PPO activity. They reported that in the extraction of PPO from the sapodilla plum, maximum PPO activity is reported when the PVP concentration is held at 3%. Meanwhile another study on the extraction of PPO from apples showed that a PVP concentration of 1% is enough to achieve maximum PPO activity level, and increasing it to 2% does not change enzyme activity.[53] In the same study by Rocha et al,[53] the effect of using either PVP or PVPP has also been studied. When PVPP is used, a higher PPO activity of 440 U/mL-min was achieved as compared to a PPO activity of 68 U/mL-min when PVP is used.

Detergents

Detergents are another class of additives that may be used in the extraction of enzymes. Detergents are polar lipid molecules that are soluble in water.[38] Each detergent molecule consists of a hydrophobic tail and a hydrophilic head. The hydrophobic tail is composed of a linear or branched hydrocarbon while the hydrophilic head may have diverse chemical structures.[41] To remove membrane-bound PPO in the plant's cell, it is recommended to use detergents to separate the enzyme from the membrane.[38,42] Detergents disrupt the structure of a biological membrane and dissolve it resulting to the solubilization of the protein.[41] Detergents then replace the membrane with the aliphatic or aromatic chains of the detergent's hydrophobic tail which then bind to the protein.[38]

In solution, detergents are characterized based on a number of properties. One property of detergents is the critical micelle concentration (CMC). At the CMC value, detergents form aggregates called micelles where the hydrophobic parts of detergent molecules cluster together at the micelle's interior and the hydrophilic parts are exposed to the surrounding aqueous medium.[41] Solubilized proteins form complexes with detergent micelles which shield the hydrophobic parts of the protein from the aqueous solvent. The number of detergent monomers in a single micelle is called the aggregation number. Solution above its CMC value will have both detergent micelles and monomers in equilibrium with each other.[38] Another detergent property is the micelle molecular weight. A micelle formed by a particular detergent has a particular molecular weight. The aggregation number is the ratio of the micelle molecular weight and the monomer molecular weight. A detergent can also be characterized by its own hydrophile-lipophile balance (HLB). The value of the HLB indicates the overall hydrophilic properties of a detergent. It can also be used to determine the denaturing property of a detergent. Detergents with HLB values greater than 7 are more soluble in aqueous solutions than in organic solutions.[38] Those with HLB values within 12 and 16 are relatively non-denaturing detergents while those with values above 20 are generally denaturing. The detergent's cloud point is the temperature where the micelles undergo phase separation. This property is used for protein purification.

The three types of detergents are ionic (either anionic or cationic), non-ionic (uncharged), and zwitterionic (with both positively and negatively charged groups but with a net charge of zero).[37] A list of common laboratory detergents and their properties are listed in Table 2. Among these, ionic detergents, although effective solubilizers, are not used in enzyme extraction as they denature proteins.[39] Non-ionic detergents, on the other hand, are widely used in membrane solubilization as they have a mild effect on the enzyme structure and activity.[38] Finding the best detergent to solubilize a particular enzyme is still largely empirical.[38,41] A prime consideration in detergent selection is its ability to maintain enzyme activity. A detergent's compatibility with downstream processes and biochemical assays also has to be taken into account. Triton X-100, for example, interferes with UV absorption processes due to the presence of aromatic rings in their structure which absorb UV light at 280 nm wavelength.[41]

Triton X-100, a non-ionic detergent commonly used as an additive in enzyme extraction, can provide a mild, nondenaturing condition during extraction.[61] The addition of a minimal amount of Triton X-100 (e.g. 0.01% (v/v)) can also prevent the irreversible binding of the protein to the materials it may come in contact with such as glass and plastic.[36] Most proteins can also tolerate Triton X-100 concentrations of up to 3% w/v and still display complete activity.[38,62] There are different detergents available and the best one to use for the extraction of a particular enzyme has to be determined empirically.[36] However, the use of some detergents, like Triton X-100, is not compatible with ammonium sulphate precipitation and gel filtration chromatography. The use of Triton X-100 alongside ammonium sulphate precipitation would separate the detergent as a floating layer. This layer would contain the protein to be extracted resulting to no separation of the target enzyme. For gel filtration chromatography, Triton X-100 would remain with the protein producing the same result.[62]

In Table 1, there is one study which added Triton X-100 in the extraction buffer. Research studies have also reported the effect of Triton X-100 in increasing the PPO activity. Keeping all variables constant, adding 0.25% Triton X-100 to the extraction medium greatly increased the activity of the PPO extracted from the apple fruit by as much as 13810 U/min-mL.[53] Similarly, in the extraction of PPO from the raspberry fruit, the PPO activity also increased from 0.125 ΔOD/min-g to 0.690 ΔOD/min-g upon the addition of 0.5% Triton X-100.[45] These results are also in consonance when banana roots are the PPO source. The PPO activity increased from

Table 2. Common Laboratory Detergents and Their Properties[41]

Detergent	Critical Micelle Concentration (mM) at 25°C	Aggregation Number	Molecular Weight (Da)	Cloud point (°C) in Low-Salt Buffers
Nonionic detergents				
Triton X-100	0.17-0.3	100-150	630 (ave)	64-65
Triton X-114	0.2-0.35	-	540 (ave)	20-25
Tween-20	0.059	-	1230 (ave)	76
Tween-80	0.012	58	1310 (ave)	93
Zwitterionic detergents				
CHAPS	6.5	4-14	614.9	
SB-10	25-40	41	307.6	
Ionic detergents				
Cholate, sodium salt	9-15	2	430.6	
SDS	7-10	62	288.4	
DTAB	15	70	280.3	

SDS, Sodium dodecyl sulfate**; DTAB,** dodecyltrimethylammonium bromide

221 U/s-g to 396 U/s-g when 0.25% Triton X-100 is added to the extraction buffer.[46] These studies show that the detergent has a positive effect on the enzyme activity by successful solubilization of membrane-bound PPOs in the plant.

Characterization of PPO

PPOs isolated from different plant sources can also be distinguished based on a variety of characteristics. The PPO's activity as a function of pH, temperature, and type of substrate it catalyzes are the most common characteristics investigated. The studies investigating the effect of temperature and pH on the PPO activity of different plants including plant pulp, kernel, and seed sources are presented in Table 3. As shown in Table 3, temperature and pH are two factors that play an important role in enzyme activity. The values of these two factors that lead to the maximum activity of the enzyme are considered as optimal values.

Temperature

Temperature is an important factor that affects the PPO activity. The forces holding the protein together (hydrogen boding, ionic bridging, van der Waals interactions, and hydrophobic interactions) and are responsible for the tertiary structure of a protein are weak and can easily be broken down by an increase in heat.[36,63] This is why enzymes are naturally thermosensitive. Typically, the activity of an enzyme increases with increasing temperature, reaches a maximum value, and declines with further increase in temperature.[2] The temperature value where the enzyme activity is maximum is also referred to as the optimum temperature. A semblance to a bell-shaped curve is produced when this temperature dependence of enzyme activity is plotted.[63] This is because the velocity of chemical reactions increases with temperature by a factor of 2-3 for every 10°C according to the Van 't Hoff rule.[50] However, when the temperatures are raised to a certain high temperature, enzyme denaturation occurs.[63] Therefore, the increase in catalytic efficiency with increasing temperature is being competed with enzyme denaturation at high temperatures.[64] The temperature where denaturation occurs is characteristic for a particular enzyme. Some enzymes are sensitive to temperature while thermophilic enzymes are stable even at extremely high temperatures.

The so-called optimum temperature of an enzyme is also not constant as the enzyme denatures while being incubated. The denaturation of an enzyme is a time-dependent process which usually follows first-order kinetics.[2,26] This implies that the enzyme activity taken immediately after extraction is considerably higher than when its activity is taken after incubation at the same temperature after a longer period of time.[50]

As seen in Table 3, the temperature range where PPO displays enzyme activity and the optimum temperature within this temperature range are largely variable and dependent on the plant source. From the same table, it can be seen that even PPOs from the same fruit but of different varieties display different optimum temperatures. The optimum temperature of PPO from Manila mango and Tainong mango are 65°C and 30°C, respectively.

pH

Just like temperature, a plot of enzyme activity as a function of pH normally resembles that of a bell-shaped curve.[63] The pH value where enzyme activity is observed to be at the maximum value is called the optimum pH. This pH value is usually chosen as a standard pH in assays of the enzyme.[2] It is worth noting that the pH at which an enzyme shows maximum activity in vitro may be different from the pH of the enzyme's environment in vivo.[37] Many enzymes have optimum pH values within the neutral range (between pH 7-8) although some enzymes like pepsin and alkaline phosphatase have optimum pH values of 2 and 10.5, respectively, which are very far from the neutral pH range.[63]

Most enzymes undergo irreversible denaturation in solutions with very high and very low pH through the attack on the enzyme's tertiary structure.[5,63] The state of protonation of the amino acids' functional groups as well as the enzyme's three-dimensional structure affect the enzyme activity.[65] The pH influences the enzyme activity by affecting the ionization of the prototropic groups (e.g. carboxyl group, imidazolium group, sulfhydryl group) in the enzyme's active site.[5] These prototropic groups are located in the side chains of the amino acid residues constituting the enzyme. They may be involved in maintaining the active site's proper conformation, in the binding of substrate to the enzyme, or in transforming the substrate to its products.[5] Moreover, pH can change the charged states of both substrate and enzyme which can affect the binding of the substrate on the enzyme and its catalysis.[2,66]

The optimum pH of different plant PPOs was shown to vary from pH 4-8.[2] The pH optimum varies widely with plant source as shown in Table 3. This may be due to the differences in amino acid sequences of PPOs taken from different sources. At different pH values, the state of protonation of the ionizable amino acid residues affect intermolecular interactions. This would translate to varied conformational changes of an enzyme under different pH values.[2,67]

Similar to temperature, optimum pH is also a function of the substrate and purification methods employed.[5] As shown in Table 3, PPO from field bean has an optimum pH of 4.0 when catechol and 4-methylcatechol are used as substrates but its optimum pH changes to 5.0 when DOPA is used as substrate.[68] This may be due to the different binding capacity of the substrates to the active site under acidic and alkaline conditions.[2]

Substrate Specificity

The primary substrates of PPO are various phenolic compounds. Just like any other enzyme, PPO tend to be substrate specific. PPOs from different plant sources show different enzymatic activity on different substrates. PPOs exhibit high activities for substrates that show high affinity or preference towards it.[2] The structure of the substrate (nature of the side chains, number of hydroxyl groups, and their positions) has a significant effect on the enzyme activity. Aside from the type of plant, the enzyme's specificity toward

Table 3. Effect of Temperature and pH on the Activity of PPO

Source	Substrate	Buffer/s Used for pH Effect	Buffer Used for Temp. Effect	Process Conditions (Optimized Setting)		Reaction Time	PPO Activity at Optimum pH & Temp. (U/mg)	Reference
				pH	Temp. (°C)			
Fruit pulp/meat								
Manila mango	0.05 M catechol	0.1 M citric acid-0.2 M sodium phosphate	0.1 M citric acid-0.2 M sodium phosphate	3.0-7.0 (6.0)	20-80 (65)	30 min	-	61
Banana	0.005 M tert-butylcatechol	0.01 M sodium acetate (pH 3-5.5), 0.01 M sodium phosphate (5.5-7.5)	-	3.0-7.5 (5.5)	-	-	-	71
Tainong mango	0.1 M catechol	0.1 M acetate (pH 3-6), 0.1 M phosphate (pH 7-9)	0.1 M phosphate	3.0-9.0 (7.0)	50-80 (30)	10 min	-	52
Kernel								
Nigerian Mango	0.5 M catechol	0.01 M phosphate	0.01 M phosphate	4.5-8.0 (6.0)	10-40 (25)	15 min	-	73
"African mango" kernel	0.01 M DOPA	0.1 M acetate (pH 4-5), 0.1 M phosphate (pH 6-7), 0.1 M Tris-HCl (pH 8-9)	0.1 M phosphate	4.0-9.0 (7.0)	30-80 (60)	10 min	-	74
Chestnut	0.09 M catechol	0.025 M potassium phosphate	0.025 M potassium phosphate	4.0-8.0 (5.0)	30-60 (40)	10 min	2.72×10^3, -	79
Chestnut	0.1 M catechol	0.05 M Na_2HPO_4-citric acid (pH 3-8), 0.05 glycine-NaOH (pH 9-10)	0.05 M sodium phosphate	3.0-10.0 (7.0)	30-80 (40)	-	363, 340	76
Seed								
Vanilla bean	0.02 M catechol	0.05 M citrate (pH 3-7), 0.1 M phosphate (pH 6-7)	0.1 M acetate	3.0-7.0 (3.4)	20-60 (37)	10 min	-	78
Field bean	0.02 M 4-methylcatechol	Glycine-HCl (pH 2.5-3.5), sodium acetate (pH 3.5-6), sodium phosphate (pH 6-8)	-	3.0-7.0 (3.0)	20-60 (37)	3 min	-	68
	0.05 M catechol			2.5-8.0 (4.0)	-			
	0.05 M 4-methylcatechol			2.5-8.0 (4.0)	-	-		
	0.01 M DOPA			2.5-8.0 (5.0)	-	-		
Pinhao seed	0.09 M catechol	0.1 M sodium citrate-phosphate (pH 4-5), 0.1 M sodium phosphate (pH 6-7), 0.1 M Tris-HCl (pH 8-9)	0.1 M sodium phosphate	4.0-9.0 (5.0)	15-50 (30)	1 min	-	75
Lotus seed	0.2 M catechol	Glycine-HCl (pH 2-3), acetic acid (pH 4-5), phosphate (pH 6-8), glycine-NaOH (pH 9-10), carbonate (pH 11)	0.2 M phosphate	2.0-11.0 (7.0)	5-80 (20)	3 min	-	69
Sunflower seed	0.01 M gallic acid	0.05 M pyrophosphate	0.05 M pyrophosphate	2.0-11.0 7.9	45-100 (45)	-	-	80

For all pertinent studies, one unit of PPO activity is defined as the change in absorbance of 0.001 per minute.

substrates is influenced by the plant cultivar from which the enzymes were taken.[2]

Table 4 shows the relative activity of PPO taken from various plant sources on different substrates. As depicted, PPO from different sources have preferred substrates. The field bean PPO has high specificity towards the di-phenols catechol and 4-methylcathecol than the triphenol pyrogallol while the sunflower seed PPO has higher activity towards triphenols gallic acid and pyrogallol. For field bean, it may be concluded that the bean's active site appears to be small and can only accommodate relatively small substrates like catechol and 4-methylcatechol. This is also the reason why field bean PPO has no affinity towards bulkier substrates like gallic acid and caffeic acid.[68]

Kinetic Parameters

For enzyme-catalyzed reactions, a plot of reaction velocity (or rate of reaction) with substrate concentration yields a curve which can be broken down into three distinct regions.[69] At low substrate concentrations, there is a linear relationship between substrate and reaction velocity. At very high substrate concentrations, the curve plateaus and the reaction velocity has reached its maximum value. Between these two regions, there is a curvilinear relationship between the reaction velocity and substrate concentration.

The plot of reaction velocity and substrate concentration for a single substrate reaction follows the enzyme kinetics developed by Michaelis and Menten (see equation 2).

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

where v is the reaction velocity, V_{max} is the maximum reaction velocity, K_m is the Michaelis-Menten constant, and $[S]$ is the substrate concentration.

Equation 2 defines two kinetic parameters that are used to describe the kinetics of an enzymatic reaction: (1) the Michaelis-Menten constant, K_m, and (2) the maximum reaction velocity, V_{max}. By definition, K_m represents the substrate concentration at which half of the maximum number of active sites in the sample is filled with the substrate. For simple reactions, the value of K_m is shown to be equal to the value of the dissociation constant of the enzyme-substrate

Table 4. Substrate Specificity of PPO From Different Plant Sources

Source	Substrate	Amount of Enzyme Used	Reference Substrate	Relative Activity (%)	Reference
Manila mango pulp	0.01 M pyrogallol	33 µL per 1 mL of assay mixture	0.01 M catechol	145	61
	0.01 M catechol			100	
	0.01 M 4-methylcatechol			62	
	0.01 M DL-DOPA			19	
Papaya	0.5 M catechol	0.25 mL per 3 mL of assay mixture	0.5 M catechol	100	81
	0.06 M 4-methylcatechol			47.6	
	0.015 M L-DOPA			6.4	
	0.003 M catechin			0	
Field bean seed	0.01 M catechol	10-100 µg per 1 mL of assay mixture	0.01 M catechol	100	68
	0.01 M 4-methyl catechol			140	
	0.01 M pyrogallol			24	
	0.01 M L-DOPA			22.6	
	0.01 M tyrosine			0	
	0.01 M gallic acid			0	
	0.01 M caffeic acid			0	
Chestnut	0.03 M catechol	0.1 mL per 3 mL of assay mixture	0.03 M catechol	100	79
	0.03 M pyrogallic acid			83.72	
	0.03 M cresol			0	
	0.03 M tyrosine			0	
Sunflower seed	3.33 mM gallic acid	0.05 mL per 3 mL of assay mixture	3.33 mM gallic acid	100	80
	0.07 mM pyrogallol			100	
	0.04 mM caffeic acid			87.3	
	0.04 mM chlorogenic acid			32.3	
	3.33 mM hydroquinone			10.9	
	3.33 mM L-DOPA			8.0	
	3.33 mM pyrocatechol			0	
	3.33 mM *p*-cresol			0	
	3.33 mM tyrosine			0	

Table 5. Kinetic Parameters of Polyphenol Oxidase From Different Plant Sources

Source	Substrate	Buffer	Temperature (°C)	pH	Time (min)	Conc. Range Studied (mM)	K_m (mM)	V_{max}	Reference
Mango kernel	L-DOPA	0.1 M phosphate	-	6.8	-	-	2.55	0.0415 U/min	74
Mango pulp	Catechol	0.2 M sodium phosphate	65	7	-	1-10	15.1	-	61
Mango pulp	Catechol	0.1 M phosphate	30	7	10	10-100	6.3	256.28 U/min	52
Chestnut	Catechol	0.025 M potassium phosphate	40	7	-	-	14.30	222 U/ml	79
Borage	Catechol	0.1 M phosphate	-	7	-	1-10	2.24	5.5 mM/min	82
Gooseberry fruit	Chlorogenic acid	0.2 M phosphate	-	6	-	0.2-4	0.56	53.15 U/ml-min	83
Lotus seed	Catechol	0.2 M phosphate	-	7	-	-	6.04	416.67 U	69
Honeydew peach	Epicatechin	0.05 M phosphate	25	6.8	-	-	0.12	347 U/min	84
Chinese cabbage	Pyrogallol	0.2 M sodium phosphate	30	6	5	10-100	15.4	14.1 OD/min	21
Blueberry	Catechol	0.1 M citrate	25	6.3	3	10-500	15	2.57 ΔA/min	85
Walnut leaves	L-tyrosine	0.05 M sodium phosphate	25	6.5	-	0.29-1.96	1.9	0.08 mM/min	86
Artichoke flesh	Catechol	Phosphate	30	8	2	0.8-10	4.03	71402 U/min-ml	87

For all pertinent studies, one unit of PPO activity is defined as the change in absorbance of 0.001 per minute.

complex.[66] Therefore, K_m can be thought of a measure of the affinity of the enzyme for the particular substrate. The enzyme's substrate binding affinity decreases as K_m increases.[65] On the other hand, the V_{max} is the maximum reaction velocity (or saturation velocity) that can be achieved by an enzymatic reaction. At V_{max}, all of the enzyme's active sites are completely filled with the substrate.

To evaluate the kinetic parameters K_m and V_{max}, equation 2 can be linearized in double-reciprocal form (see equation 3) which is also called as the Lineweaver-Burke equation. A plot of $1/v$ with $1/[S]$ is sketched wherein the linear trendline that will be formed has a y-intercept of $1/V_{max}$ and a slope of K_m / V_{max}.

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S]} \quad (3)$$

It is good to note that although the kinetic parameters can be determined using the Lineweaver-Burke equation, this equation is not the only method for determining the kinetic parameters.[65] Other equations like the Eadie-Hofstee and Hanes-Woolf equations can also be used for enzyme kinetics. Non-linear regression can also be employed for the determination of the kinetic parameters using various commercially available software (e.g. SigmaPlot).

Table 5 shows the kinetic parameters K_m and V_{max} of PPO extracted and characterized from different plant sources. As it can be seen in this table, most of the studies used catechol as a substrate. The concentration ranges used are also varied from as low as 0.2 mM to as high as 500 mM.

Conclusions

There are several factors that can affect PPO extraction from different plant sources. These factors include the pH of the extraction mixture, extraction temperature, type of buffer, mass to solvent, extraction time, and the used additives (e.g. protease inhibitors, protective agents, and detergents). The pH of the extraction mixture could be in the range of 6.0–8.0, preferably at pH 7.0. In order to avoid denaturation and minimize protease activity, the extraction temperature should be kept low (in the range of 0 – 4°C). The type of buffer, mass to solvent, extraction time, and additives used for PPO extraction depend on the plant materials source, characteristics of the enzyme, and the subsequent purification steps. The PPO isolated from different plant sources show varied optimum pH, optimum temperature, substrate affinity, and kinetic parameter values which make the PPO enzymes distinguishable from one another.

Authors' Contributions

Both authors contributed equally to current study.

Conflict of Interest Disclosures

The authors declare that they have no conflict interests.

References

1. Yada RY, Jackman RL, Smith JL. Protein Structure-Function Relationships in Foods. New York: Springer Science & Business Media; 1994.
2. Jukanti A. Polyphenol Oxidases (PPOs) in Plants. Singapore: Springer Nature; 2017.
3. Nicolas JJ, Richard-Forget FC, Goupy PM, Amiot MJ, Aubert SY. Enzymatic browning reactions in apple and apple products. Crit Rev Food Sci Nutr. 1994;34(2):109-157. doi:10.1080/10408399409527653.
4. Yoruk R, Marshall MR. Physicochemical properties and function of plant polyphenol oxidase: a review. J Food Biochem. 2003;27(5):361-422. doi:10.1111/j.1745-4514.2003.tb00289.x.
5. Whitaker JR. Principles of Enzymology for the Food Sciences. 2nd ed. New York: Marcel Dekker; 1994.

6. Eskin NM, Shahidi F. Biochemistry of Foods. 3rd ed. Elsevier Inc; 2013.
7. Vámos-Vigyázó L. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit Rev Food Sci Nutr. 1981;15(1):49-127. doi:10.1080/10408398109527312.
8. Colak A, Sahin E, Yildirim M, Sesli E. Polyphenol oxidase potentials of three wild mushroom species harvested from Lişer High Plateau, Trabzon. Food Chem. 2007;103(4):1426-1433. doi:10.1016/j.foodchem.2006.10.059.
9. Espín JC, Morales M, García-Ruiz PA, Tudela J, García-Cánovas F. Improvement of a continuous spectrophotometric method for determining the monophenolase and diphenolase activities of mushroom polyphenol oxidase. J Agric Food Chem. 1997;45(4):1084-1090. doi:10.1021/jf960428a.
10. Kanda K, Sato T, Ishii S, Enei H, Ejiri S. Purification and properties of tyrosinase isozymes from the gill of Lentinus edodes fruiting body. Biosci Biotechnol Biochem. 1996;60(8):1273-1278. doi:10.1271/bbb.60.1273.
11. Güray MZ, Şanlı-Mohamed G. A new thermophilic polyphenol oxidase from *Bacillus* sp.: partial purification and biochemical characterization. J Proteins Proteom. 2013;4(1):11-20.
12. Bartolo I, Birk EO. Some factors affecting Norway lobster (*Nephrops norvegicus*) cuticle polyphenol oxidase activity and blackspot development. Int J Food Sci Technol. 1998;33(3):329-336. doi:10.1046/j.1365-2621.1998.00168.x.
13. Chen JS, Balaban MO, Wei CI, Gleeson RA, Marshall MR. Effect of carbon dioxide on the inactivation of florida spiny lobster polyphenol oxidase. J Sci Food Agric. 1993;61(2):253-259. doi:10.1002/jsfa.2740610219.
14. Simpson BK, Marshall MR, Otwell WS. Phenol oxidase from shrimp (*Penaeus setiferus*): purification and some properties. J Agric Food Chem. 1987;35(6):918-921. doi:10.1021/jf00078a017.
15. Zamorano JP, Martínez-Álvarez O, Montero P, Gómez-Guillén MDC. Characterisation and tissue distribution of polyphenol oxidase of deepwater pink shrimp (*Parapenaeus longirostris*). Food Chem. 2009;112(1):104-111. doi:10.1016/j.foodchem.2008.05.061.
16. Cortez JT, Herrera-Mendez CH, Sauri-Duch E, de Lourdes Vargas y Vargas M, Solís-Pereira S. Purification and partial characterization of polyphenol oxidase from sapodilla plum (*Achras sapota*). Food Nutr Sci. 2013;4(7):727-734. doi:10.4236/fns.2013.47093.
17. Liu F, Zhao JH, Gan ZL, Ni YY. Comparison of membrane-bound and soluble polyphenol oxidase in Fuji apple (*Malus domestica* Borkh. cv. Red Fuji). Food Chem. 2015;173:86-91. doi:10.1016/j.foodchem.2014.09.169.
18. Espín JC, Trujano MF, Tudela J, García-Cánovas F. Monophenolase activity of polyphenol oxidase from Haas avocado. J Agric Food Chem. 1997;45(4):1091-1096. doi:10.1021/jf9605815.
19. Mdluli KM. Partial purification and characterisation of polyphenol oxidase and peroxidase from marula fruit (*Sclerocarya birrea* subsp. Caffra). Food Chem. 2005;92(2):311-323. doi:10.1016/j.foodchem.2004.07.026.
20. Marri C, Frazzoli A, Hochkoeppler A, Poggi V. Purification of a polyphenol oxidase isoform from potato (*Solanum tuberosum*) tubers. Phytochemistry. 2003;63(7):745-752. doi:10.1016/s0031-9422(03)00353-4.
21. Nagai T, Suzuki N. Partial purification of polyphenol oxidase from Chinese cabbage *Brassica rapa* L. J Agric Food Chem. 2001;49(8):3922-3926. doi:10.1021/jf000694v.
22. Yang CP, Fujita S, Kohno K, Kusubayashi A, Ashrafuzzaman M, Hayashi N. Partial purification and characterization of polyphenol oxidase from banana (*Musa sapientum* L.) peel. J Agric Food Chem. 2001;49(3):1446-1449. doi:10.1021/jf001051i.
23. Rahman AN, Ohta M, Nakatani K, Hayashi N, Fujita S. Purification and characterization of polyphenol oxidase from cauliflower (*Brassica oleracea* L.). J Agric Food Chem. 2012;60(14):3673-3678. doi:10.1021/jf300380b.
24. Benaceur F, Chaibi R, Berrabah F, et al. Purification and characterization of latent polyphenol oxidase from truffles (*Terfezia arenaria*). Int J Biol Macromol. 2020;145:885-893. doi:10.1016/j.ijbiomac.2019.09.126.
25. Park YK, Sato HH, Almeida TD, Moretti RH. Polyphenol oxidase of mango (*Mangifera indica* var. Haden). J Food Sci. 1980;45(6):1619-1621. doi:10.1111/j.1365-2621.1980.tb07575.x.
26. Robinson SP, Loveys BR, Chacko EK. Polyphenol oxidase enzymes in the sap and skin of mango fruit. Funct Plant Biol. 1993;20(1):99-107. doi:10.1071/pp9930099.
27. Saby John K, Bhat SG, Prasada Rao UJ. Isolation and partial characterization of phenol oxidases from *Mangifera indica* L. sap (latex). J Mol Catal B Enzym. 2011;68(1):30-36. doi:10.1016/j.molcatb.2010.09.004.
28. Crecchio C, Ruggiero P, Pizzigallo MD. Polyphenoloxidases immobilized in organic gels: properties and applications in the detoxification of aromatic compounds. Biotechnol Bioeng. 1995;48(6):585-591. doi:10.1002/bit.260480605.
29. Edwards W, Bownes R, Leukes WD, et al. A capillary membrane bioreactor using immobilized polyphenol oxidase for the removal of phenols from industrial effluents. Enzyme Microb Technol. 1999;24(3-4):209-217. doi:10.1016/s0141-0229(98)00110-0.
30. Shao J, Huang LL, Yang YM. Immobilization of polyphenol oxidase on alginate–SiO2 hybrid gel: stability and preliminary applications in the removal of aqueous phenol. J Chem Technol Biotechnol. 2009;84(4):633-635. doi:10.1002/jctb.2086.
31. Wang H, Li S, Li J, Zhong L, Cheng H, Ma Q. Immobilized polyphenol oxidase: preparation, optimization and oxidation of phenolic compounds. Int J Biol Macromol. 2020;160:233-244. doi:10.1016/j.ijbiomac.2020.05.079.
32. Mukherjee S, Basak B, Bhunia B, Dey A, Mondal B. Potential use of polyphenol oxidases (PPO) in the bioremediation of phenolic contaminants containing industrial wastewater. Rev Environ Sci Biotechnol. 2013;12(1):61-73. doi:10.1007/s11157-012-9302-y.
33. Mohammadi A, Bayandori Moghaddam A, Dinarvand R, Rezaei-Zarchi S. Direct electron transfer of polyphenol oxidase on carbon nanotube surfaces: application in biosensing. Int J Electrochem Sci. 2009;4(7):895-905.
34. Schmidt JC. Enzyme-based electrodes for environmental monitoring applications. Field Analytical Chemistry & Technology. 1998;2(6):351-361. doi:10.1002/(sici)1520-6521(1998)2:6<351::aid-fact5>3.0.co;2-r.
35. Shan D, Mousty C, Cosnier S. Subnanomolar cyanide detection at polyphenol oxidase/clay biosensors. Anal Chem. 2004;76(1):178-183. doi:10.1021/ac034713m.
36. Bonner PLR. Protein Purification. 1st ed. Taylor & Francis; 2007.
37. Bonner PLR. Protein Purification. 2nd ed. Taylor & Francis; 2018.
38. Rosenberg IM. Protein Analysis and Purification: Benchtop

Techniques. 2nd ed. Boston: Birkhäuser; 2005.
39. Ahmed H. Principles and Reactions of Protein Extraction, Purification, and Characterization. Florida: CRC Press LLC; 2005.
40. Hatti-Kaul R, Mattiasson B. Isolation and Purification of Proteins. New York: Marcel Dekker Inc; 2003.
41. Burgess RR, Deutscher MP. Guide to Protein Purification. 2nd ed. Academic Press; 2009.
42. Coligan JE, Dunn BM, Speicher DW, Wingfield PT. Current Protocols in Protein Science. John Wiley & Sons Inc; 2007.
43. Cutler P. Protein Purification Protocols. 2nd ed. New Jersey: Humana Press Inc; 2004.
44. Eisenthal R, Danson MJ. Enzyme Assays: A Practical Approach. 2nd ed. New York: Oxford University Press; 2002.
45. González EM, de Ancos B, Cano MP. Partial characterization of polyphenol oxidase activity in raspberry fruits. J Agric Food Chem. 1999;47(10):4068-4072. doi:10.1021/jf981325q.
46. Wuyts N, De Waele D, Swennen R. Extraction and partial characterization of polyphenol oxidase from banana (*Musa acuminata* Grande naine) roots. Plant Physiol Biochem. 2006;44(5-6):308-314. doi:10.1016/j.plaphy.2006.06.005.
47. Tsai DS, Arteca RN, Bachman JM, Phillips AT. Purification and characterization of 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyls. Arch Biochem Biophys. 1988;264(2):632-640. doi:10.1016/0003-9861(88)90329-3.
48. Salis A, Bilanicova D, Ninham BW, Monduzzi M. Hofmeister effects in enzymatic activity: weak and strong electrolyte influences on the activity of *Candida rugosa* lipase. J Phys Chem B. 2007;111(5):1149-1156. doi:10.1021/jp066346z.
49. Salis A, Monduzzi M. Not only pH. Specific buffer effects in biological systems. Curr Opin Colloid Interface Sci. 2016;23:1-9. doi:10.1016/j.cocis.2016.04.004.
50. Bisswanger H. Practical Enzymology. 2nd ed. Weinheim: Wiley-VCH Verlag & Co; 2011.
51. Good NE, Winget GD, Winter W, Connolly TN, Izawa S, Singh RM. Hydrogen ion buffers for biological research. Biochemistry. 1966;5(2):467-477. doi:10.1021/bi00866a011.
52. Wang J, Jiang W, Wang B, Liu S, Gong Z, Luo Y. Partial properties of polyphenol oxidase in mango (*Mangifera indica* L. cv."Tainong") pulp. J Food Biochem. 2007;31(1):45-55. doi:10.1111/j.1745-4514.2007.00097.x.
53. Rocha AM, Cano MP, Galeazzi MA, Morais AM. Characterisation of 'Starking' apple polyphenoloxidase. J Sci Food Agric. 1998;77(4):527-534. doi:10.1002/(sici)1097-0010(199808)77:4<527::aid-jsfa76>3.0.co;2-e.
54. Jukanti AK, Bruckner PL, Habernicht DK, Foster CR, Martin JM, Fischer AM. Extraction and activation of wheat polyphenol oxidase by detergents: biochemistry and applications. Cereal Chem. 2003;80(6):712-716. doi:10.1094/cchem.2003.80.6.712.
55. Mukherjee S, Bandyopadhayay B, Basak B, Mandal N, Apurba DE, Mondal B. An improved method of optimizing the extraction of polyphenol oxidase from potato (*Solanum tuberosum* L.) peel. Not Sci Biol. 2012;4(1):98-107. doi:10.15835/nsb417186.
56. Ahmad MN, Liew SL, Yarmo MA, Said M. Optimization of protease extraction from horse mango (*Mangifera foetida* Lour) kernels by a response surface methodology. Biosci Biotechnol Biochem. 2012;76(8):1438-1444. doi:10.1271/bbb.120073.
57. Ajila CM, Bhat SG, Prasada Rao UJ. Valuable components of raw and ripe peels from two Indian mango varieties. Food Chem. 2007;102(4):1006-1011. doi:10.1016/j.foodchem.2006.06.036.
58. Arshad ZI, Amid A, Yusof F, Jaswir I, Ahmad K, Loke SP. Bromelain: an overview of industrial application and purification strategies. Appl Microbiol Biotechnol. 2014;98(17):7283-7297. doi:10.1007/s00253-014-5889-y.
59. Mehrnoush A, Mustafa S, Sarker MZ, Yazid AM. Optimization of serine protease purification from mango (*Mangifera indica* cv. Chokanan) peel in polyethylene glycol/dextran aqueous two phase system. Int J Mol Sci. 2012;13(3):3636-3649. doi:10.3390/ijms13033636.
60. Mayer AM, Harel E. Polyphenol oxidases in plants. Phytochemistry. 1979;18(2):193-215. doi:10.1016/0031-9422(79)80057-6.
61. Palma-Orozco G, Marrufo-Hernández NA, Sampedro JG, Nájera H. Purification and partial biochemical characterization of polyphenol oxidase from mango (*Mangifera indica* cv. Manila). J Agric Food Chem. 2014;62(40):9832-9840. doi:10.1021/jf5029784.
62. Scopes RK. Protein Purification: Principles and Practice. 3rd ed. New York: Springer-Verlag; 1994.
63. Bisswanger H. Enzyme assays. Perspect Sci. 2014;1(1-6):41-55. doi:10.1016/j.pisc.2014.02.005.
64. Li F. Purification, kinetic parameters, and isoforms of polyphenol oxidase from "Xushu 22" sweet potato skin. J Food Biochem. 2020;44(11):e13452. doi:10.1111/jfbc.13452.
65. Copeland RA. Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis. 2nd ed. New York: John Wiley & Sons; 2000.
66. Lv Y, Cai L, Yang M, Liu X, Hui N, Li J. Purification, characterisation, and thermal denaturation of polyphenoloxidase from prawns (*Penaeus vannamei*). Int J Food Prop. 2017;20(Suppl 3):S3345-S3359. doi:10.1080/10942912.2017.1354019.
67. Ben-Shalom N, Kahn V, Harel E, Mayer AM. Catechol oxidase from green olives: properties and partial purification. Phytochemistry. 1977;16(8):1153-1158. doi:10.1016/s0031-9422(00)94350-4.
68. Paul B, Gowda LR. Purification and characterization of a polyphenol oxidase from the seeds of field bean (*Dolichos lablab*). J Agric Food Chem. 2000;48(9):3839-3846. doi:10.1021/jf000296s.
69. Cai XX, Hong YX, Wang SY, Zhao LN, Rao PF. Purification and enzymatic characteristics of a novel polyphenol oxidase from lotus seed (*Nelumbo nucifera* Gaertn.). Int J Food Sci Technol. 2015;50(4):1026-1032. doi:10.1111/ijfs.12735.
70. George HL, Christoffersen RE. Differential latency toward (–)-epicatechin and catechol mediated by avocado mesocarp polyphenol oxidase (PPO). Postharvest Biol Technol. 2016;112:31-38. doi:10.1016/j.postharvbio.2015.09.036.
71. Sojo MM, Nuñez-Delicado E, García-Carmona F, Sánchez-Ferrer A. Partial purification of a banana polyphenol oxidase using Triton X-114 and PEG 8000 for removal of polyphenols. J Agric Food Chem. 1998;46(12):4924-4930. doi:10.1021/jf980473d.
72. Terefe NS, Delon A, Buckow R, Versteeg C. Blueberry polyphenol oxidase: characterization and the kinetics of thermal and high pressure activation and inactivation. Food Chem. 2015;188:193-200. doi:10.1016/j.foodchem.2015.04.040.
73. Arogba SS, Ajiboye OL, Ugboko LA, Essienette SY, Afolabi PO. Properties of polyphenol oxidase in mango (*Mangifera indica*) kernel. J Sci Food Agric. 1998;77(4):459-462. doi:10.1002/(sici)1097-0010(199808)77:4<459::aid-jsfa61>3.0.co;2-o.
74. Sanni DM. Isolation, partial purification and characterization of polyphenoloxidase from two species of African Mango seeds

(*Irvingia gabonensis* and *Irvingia wombolu*). Adv Biochem. 2016;4(4):47-52. doi:10.11648/j.ab.20160404.12.
75. Daroit DJ, Corrêa APF, Klug TV, Brandelli A. Partial purification and characterization of polyphenol oxidase from *Araucaria angustifolia* (Bert, O. Ktze) seeds. J Food Biochem. 2010;34(6):1216-1230. doi:10.1111/j.1745-4514.2010.00360.x.
76. Gong Z, Li D, Liu C, Cheng A, Wang W. Partial purification and characterization of polyphenol oxidase and peroxidase from chestnut kernel. LWT Food Sci Technol. 2015;60(2 Pt 2):1095-1099. doi:10.1016/j.lwt.2014.10.012.
77. Chilaka FC, Anosike EO, Egbuna PC. Purification and properties of polyphenol oxidase from oil bean (*Pentaclethra macrophylla* Benth) seeds. J Sci Food Agric. 1993;61(1):125-127. doi:10.1002/jsfa.2740610120.
78. Waliszewski KN, Márquez O, Pardio VT. Quantification and characterisation of polyphenol oxidase from vanilla bean. Food Chem. 2009;117(2):196-203. doi:10.1016/j.foodchem.2009.03.118.
79. Xu J, Zheng T, Meguro S, Kawachi S. Purification and characterization of polyphenol oxidase from Henry chestnuts (*Castanea henryi*). J Wood Sci. 2004;50(3):260-265. doi:10.1007/s10086-003-0554-6.
80. Raymond J, Rakariyatham N, Azanza JL. Purification and some properties of polyphenoloxidase from sunflower seeds. Phytochemistry. 1993;34(4):927-931. doi:10.1016/s0031-9422(00)90689-7.
81. Cano MP, Lobo MG, de Ancos B, Galeazzi MAM. Polyphenol oxidase from Spanish hermaphrodite and female papaya fruits (*Carica papaya* cv. Sunrise, Solo Group). J Agric Food Chem. 1996;44(10):3075-3079. doi:10.1021/jf960119k.
82. Alici EH, Arabaci G. Purification of polyphenol oxidase from borage (*Trachystemon orientalis* L.) by using three-phase partitioning and investigation of kinetic properties. Int J Biol Macromol. 2016;93(Pt A):1051-1056. doi:10.1016/j.ijbiomac.2016.09.070.
83. Bravo K, Osorio E. Characterization of polyphenol oxidase from Cape gooseberry (*Physalis peruviana* L.) fruit. Food Chem. 2016;197(Pt A):185-190. doi:10.1016/j.foodchem.2015.10.126.
84. Liu L, Cao S, Yang H, Qi X. Pectin plays an important role on the kinetics properties of polyphenol oxidase from honeydew peach. Food Chem. 2015;168:14-20. doi:10.1016/j.foodchem.2014.07.064.
85. Siddiq M, Dolan KD. Characterization of polyphenol oxidase from blueberry (*Vaccinium corymbosum* L.). Food Chem. 2017;218:216-220. doi:10.1016/j.foodchem.2016.09.061.
86. Zekiri F, Molitor C, Mauracher SG, et al. Purification and characterization of tyrosinase from walnut leaves (*Juglans regia*). Phytochemistry. 2014;101:5-15. doi:10.1016/j.phytochem.2014.02.010.
87. Ziyan E, Pekyardimci Ş. Characterization of polyphenol oxidase from Jerusalem artichoke (*Helianthus tuberosus*). Turk J Chem. 2003;27(2):217-225.