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MINI REVIEW

Determination of polyphenol oxidase and peroxidase activity in plants rich in phenolics and chlorophylls

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ABSTRACT

Investigating the enzyme activities involved in the browning of plant tissues can be difficult when there are high amounts of endogenous phenols and / or there is a 'latent stage' (viz., membrane bound) of the browning enzymes polyphenol oxidases (PPOs) and peroxidases (PODs) in the crude extract. To extract or partial purify of these enzymes involves removal of phenolic metabolites and activation of the enzyme from its latent stage. The highly reactive phenolic compounds can be removed by incorporating polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP) and polyethylene glycol (PEG) into enzyme extraction solutions, thus improving enzymatic activity. PPO are present naturally in latent forms, and thus extraction of these enzymes may require the use of activation agents such as sodium dodecyl sulphate (SDS) and detergents such as Triton X (TTX). However, these detergents have disadvantages in that they also release chlorophylls, which give a dark green supernatant and later interfere with enzymatic activity. To overcome this problem, extraction of the enzymes using temperature-induced phase separation in TTX-114 was introduced. Within this review, the detailed overview of the current phytochemical approaches for extraction and purification of such enzymes are described.

Key words: latent PPO; optimisation; peripheral membrane POD; total phenol content

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INTRODUCTION

Protein extraction for PPOs and PODs has historically been difficult. This is due to secondary metabolism in the form of pigments (*e.g.,* phenols, tannins), unidentified polysaccharides and interfering proteins (Babu et al., 2008; Bru et al., 1984). Such compounds can lower extractable enzyme activity by interacting with enzymes like PPOs and PODs to produce covalent and non-covalent complexes that result in enzymatic inactivation (Bru et al., 1984; Leon et al., 2002; Sakharov et al., 2000). Extraction with buffer alone will not remove these compounds. In attempts to improve this situation, various different extraction methods have been employed involving the use of reducing agents, phenol absorbing agents and organic solvents like acetone and alcohol (Bru et al., 1984; Gonzalez et al., 1999; Gonzalez et al., 2000). However, the use of such notionally beneficial ingredients may in itself lead to enzyme inhibition, denaturisation or the inactivation of the native form of the active enzymes (Escribano et al., 2002; Nicolas et al., 1994).

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PPOs are known as integral membrane proteins (Gauillard and Richard Forget 1997; Jukanti et al., 2003; Okot-Kotber et al., 2002). Thus, extraction of these enzymes has been successfully achieved by incorporating detergents like SDS and TTX in the extraction buffer. However, these detergents also digest chloroplasts and release chlorophyll pigments which interfere with enzymatic activity (Bru et al., 1984; Sanchez-Ferrer et al., 1989a; Sanchez-Ferrer et al., 1989b). For different plant systems, the combination and concentration of such compounds needs to be optimised because of variation in the type and qualities of the specific phenols and enzymes of interest (Sanchez-Ferrer et al., 1989a). Therefore, an understanding of the function of each component is necessary to facilitate the optimisation of PPO and POD extraction protocols. A comprehensive review of the methods used to study PPO and also POD activity was made around three decades ago by Mayer (1987). Herein, the focus has been to update that information. Accordingly, this review highlights up to date aspects of assessing PPO and POD activity in plant-based sources. The focus is upon extraction and purification of PPOs for maximal activity. The function of components used in PPO and POD extractions are reviewed in consideration of their advantages and limitations.

GRINDING OF PLANT TISSUE

Grinding tissue to fine particles prior to extraction is an important starting point in enzyme extraction (Isaacson et al., 2006). Evergreen and thick plant materials can be very hard to disrupt due to a very complex assemblies of polysaccharides; e.g. cell walls (Lerouxel et al., 2006; Wei et al., 2008). Tissue grinding with liquid nitrogen using a mortar and pestle with or without acid wash sand has been used extensively. However, there attendant risk that the enzyme and other proteins of interest can be degraded during this slow and awkward process (Isaacson et al., 2006; Lerouxel et al., 2006).

REMOVAL OF PLANT SECONDARY METABOLITES

Polyvinylpolypyrrolidone (PVPP) or polyvinylpyrrolidone (PVP)

PVPP (water insoluble) and PVP (water soluble) have been used for several decades in extraction media to remove plant phenolic compounds (Hulme et al., 1964; Jones et al., 1965; Mayer and Harel 1979). The mode of action of PVPP is to remove the inhibition of enzymatic activity by certain phenols. The efficacy is attributed to the ability of PVPP to attach to the phenol moiety before it combines with the enzyme (Hulme et al., 1964) and is likely due to competitive hydrogen binding of PVPP with the phenols (Jones et al., 1965). Precipitating the PVPP-phenolic complex by centrifugation allows phenolics removal from the extract (Wei et al., 2008). PVPP at 1% has been used in the extraction mixture for many tissues, including longan (Jiang 1999), protea (Dai and Paull, 1997), and apple (Rocha et al., 1998). For plants rich in phenolic compounds such as raspberry (Gonzalez et al., 1999) and blackberry (Gonzalez et al., 2000), a higher concentration of at 4% is typically used. In addition to the use of PVPP in the extraction solution, Baruah and Mahanta (2003) ensured that remaining polyphenols were removed from tea leaf extract by passing the crude enzyme extract through a PVPP column. In the study of enzymatic browning in cut cinnamon myrtle (*Backhousia myrtifolia*), 5% PVPP was seemingly adequate in removing the phenols (Sommano, 2015a).

However, an excess of PVPP may also inhibit PPO activity through combination with enzyme-substrate complexes (Hulme et al., 1964) as has been found in experiments with banana root tissue (Wuyts et al., 2006). In apple extracts, PPO activity increased until the PVPP concentration reached 1%, beyond which concentration further PVPP addition caused a slight decrease in phenolase activity (Walker and Hulme, 1965). That is, the formation of such complexes inhibits or prevents PPO activity. Jones et al. (1965) observed that if a plant contains polymerised phenolics such as ‘tannins’, then PVPP is more likely

to form bulky insoluble complexes likely to reduce activity. Due to the solubility of PVP, it is not recommended for protein extraction. However, many researchers inadvertently confuse the use of soluble PVP with the insoluble PVPP form (Isaacson et al., 2006).

Polyethylene glycol (PEG)

Water insoluble complexes of flavonoid tannins and protein have been found to have an adverse effect on PPO activity in *Protea neriifolia* extract (Whitehead and Deswardt, 1982). Such compounds were removed by PEG in a similar manner as for PVP. Increasing PEG in the extraction medium from 1 to 1.5 % slightly improved PPO activity in artichoke and *Laurus nobilis* L. (Aydemir, 2004; Aydemir and Cinar, 2006). Sommano (2015a) advised a higher concentration of PEG of 5% was less effective in removing phenols in *B. myrtifolia* (Table 1).

An aqueous phase partitioning system (ATPSs) with PEG has been used successfully as a system that can eliminate interferences, such as by polysaccharides, pigments and interfering proteins, that otherwise lower enzyme activity (Babu et al., 2008). The system can be generated by mixing a combination of salt and polymer in water (e.g., phosphate and PEG) (Babu et al., 2008; Orenes-Pinero et al., 2006; Vaidya et al., 2006) and was successfully used in the purification of PPOs (Orenes-Pinero et al., 2006; Vaidya et al., 2006) and PODs (Leon et al., 2002). The advantages of this approach, as described by Babu et al. (2008), are its low cost, its scope for continuous operation, the fact that it can be easily scaled up, and that it is environmentally friendly. Nonetheless, ATPSs was suggested as a primary purification step that needed to be followed by a more selective chromatographic method (Vaidya et al., 2006).

Table 1. Total phenolic concentration in crude extracts from plant rich in polyphenol *Backhousia myrtifolia* in various extraction solutions.

Extraction	Total phenolics (mg g^{-1} FW GAE)
95% methanol	48.4±0.02
Sodium phosphate buffer[A]	40.3±0.09
PVPP1%[B]	23.7±0.14
PVPP5%[B]	11.0±0.06
PEG1%[B]	21.0±0.38
PEG5%[B]	21.2±0.27

Sommano (2015a). [A] 0.1 M sodium phosphate buffer pH 6.8. [B] Prepared in 0.1 M pH 6.8 phosphate buffer. Values are means (n = 3) ± standard error (SE).

Acetone extraction

Acetone precipitation has been used extensively in proteomic analysis and is well known to remove major secondary metabolites, including phenolic compounds and pigments (Wei et al., 2008; Wei et al., 2006). However, it has also been proved to have an adverse effect on the structure of enzymes, and thus can inhibit PPO activity (Bru et al., 1984; Escribano et al., 2002; Nicolas et al., 1994). Moreover, PPO extracted using acetone powder and kept overnight at 4°C experienced discolouration (Smith and Montgomery, 1985).

Temperature-induced phase partitioning with addition of TTX-114

TTX-114 can solubilise the protein bound membrane and release free chlorophylls with the same efficiency as TTX-100 (Sanchez-Ferrer et al., 1989a). Moreover, this detergent can form an aggregate at high temperature of large mixed micelles with the released chlorophylls. This is known as temperature induced phase partitioning with the addition of TTX-114 or cloud point extraction with TTX-114 (Bru et al., 1984; Sanchez-Ferrer et al., 1989a; Sanchez-Ferrer et al., 1994; Sanchez-Ferrer et al., 1989b). The clear supernatant resulting from high speed centrifugation contains the purified enzyme of interest. Chlorophyll and polyphenols remain in the dark green solution phase (Sanchez-Ferrer et al., 1989a).

SOLUBILISATION OF INTEGRAL MEMBRANE PROTEIN (PPOs)

Sodium dodecyl sulphate (SDS)

SDS is an agent commonly used in PPO extraction media for a number of plant systems, including wheat bran (Okot-Kotber et al., 2002), vanilla shoot (Debowska and Podstolski, 2001), protea leaf (Dai and Paull, 1997), pear (Gauillard and Richard Forget, 1997), apple and tobacco leaf (Broothaerts et al. 2000). Due to its anionic nature, SDS facilitates the activation of the latent PPO in plant extracts (Jukanti et al., 2003; Okot-Kotber et al., 2002).

PPOs are not in the latent condition in all plants. Accordingly, adding SDS to the extraction solution did not improve PPO activity in strawberry (Serradell et al., 2000) and litchi (Jiang et al., 1997). Upon use of SDS, Serradell et al. (2000) found a shift in the optimum pH for PPO activity in strawberry from 5.3 - 6 to 7.2. Jimenez and Garcia-Carmona (1996) determined that the SDS activation effect applied only in solutions with higher pH of >4.0.

Triton-X (TTX)

TTX is a non-ionic detergent that can solubilise membrane bound PPOs. Thus it is able to improve PPO activity, for example in banana (Wuyts et al., 2006), pear (Gauillard and Richard Forget, 1997) and wheat (Okot-Kotber et al., 2002). However, the chloroplast is also solubilised when this detergent is used, leading to free chlorophylls in the extract that can then interfere with PPO activity (Bru et al., 1984; Sanchez-Ferrer et al., 1989a; Sanchez-Ferrer et al., 1989b; Sommano, 2015b; Sommano et al., 2017).

Alcohols

The presence of a number of alcohols in organic solvents, namely ethanol, methanol, ethylene glycol, diethylene glycol, and propylene glycol, decreases catecholase reaction rates (Burton, 1994; Burton and Kirchmann, 1997). However, glycerol at low concentration was reported to activate PPO activity (Burton, 1994; Espin and Wichers, 1999; Estrada et al., 1993). Okot-Kotber

et al. (2002) found a positive activating effect of n-butanol on PPO activity in wheat extract. They explained that hydrophobic interactions may induce a conformational change in the enzyme structure, giving better substrate-enzyme binding.

Trypsin (protease)

Trypsin has been proved to be an effective heat tolerant PPO activator (Sanchez-Ferrer et al., 1989a; Tolbert, 1973). Gandia-Herrero et al., (2005) explained that the function of trypsin in increasing PPO activity was enhanced proteolysis generating a smaller soluble active form of PPO molecule (Laveda et al., 2001).

Sommano (2015a) studied the effect of different detergents and mild treatments on enzymatic browning activity from leaf of *B. myrtifolia*. Initially, she found relatively low PPO activity (~8 units/mL) in extracts from extractions with no added detergents. When detergents were included in the extraction buffer along with 5% PVPP, PPO activities improved (Table 2). SDS and n-butanol were more effective at higher concentrations of 2% w/v and 5% v/v, respectively. However, TTX-100 gave the highest activity of 230 units /L at 2% v/v and thus was the most efficient detergent per unit concentration among the detergents tested.

Acidic buffers (pH 5.8) with or without mild (sucrose, EDTA) treatments were used for POD extractions. The mild treatments did not improve activity. However, when the extraction buffer improved PPO activity (viz., 2% TTX-100 and 5% PVPP in phosphate buffer at pH 6.8) was used, POD activity was dramatically improved to 360 units/mL (Table 2).

Table 2. Effects of detergents on PPO activity and of mild extractions on POD activity on leaf of *B. myrtifolia*.

Phosphate buffer	Phenol absorbing agent	Detergents/ mild treatments	Enzymatic activity
1. PPO activity			(units mL^{-1})
pH 6.8	5 % (w/v) PVPP	none	7.67 ± 0.3
pH 6.8	5 % (w/v) PVPP	1 % (w/v) SDS	10.0 ± 1.7
pH 6.8	5% (w/v) PVPP	2 % (w/v) SDS	19.3 ± 0.7
pH 6.8	5 % (w/v) PVPP	5 % (w/v) SDS	24.0 ± 1.5
pH 6.8	5 % (w/v) PVPP	1% (v/v) *n*-butanol	n/d
pH 6.8	5% (w/v) PVPP	2% (v/v) *n*-butanol	19.0 ± 0.6
pH 6.8	5 % (w/v) PVPP	5% (v/v) *n*-butanol	74.7 ± 3.2
pH 6.8	5 % (w/v) PVPP	1% (v/v) TTX-100	33.0 ± 1.0
pH 6.8	5% (w/v) PVPP	2% (v/v) TTX-100	205± 9.9
pH 6.8	5 % (w/v) PVPP	5% (v/v) TTX-100	63.3 ± 0.3
pH 6.8	5% (w/v) PVPP	2% (v/v) TTX-100 (repeat)	232.6 ± 3.8

2. POD activity

pH 5.8	5 % (w/v) PVPP	none	0.0089 ± 0.0005
pH 5.8	5 % (w/v) PVPP	20% (w/v) sucrose	0.0093 ± 0.005
pH 5.8	5% (w/v) PVPP	0.1 M EDTA	0.003 ± 0.001
pH 6.8	5 % (w/v) PVPP	2% (v/v) Triton x-100	359.8 ± 63.8

Sommano (2015a). n/d = not detected. Values are means (n = 3) ± SE.

FUTHER ENZYMATIC PURIFICATION

Ammonium sulphate precipitation

Ammonium sulphate salt precipitation is generally used for partially removing inactive protein, including for the separation of the plant proteins PPOs and PODs (Escribano et al., 2002; Gauillard and Richard Forget, 1997; Jiang, 1999; Kader et al., 1997). The amount of ammonium sulphate added depends on the size of the protein of interest. For example, PPOs and PODs are usually precipitated between 45 – 85 % salt saturation (Deepa and Arumughan 2002; Escribano et al. 2002). However, ammonium sulphate then needs to be removed from the precipitants by dialysis against the same buffer used for the extraction because it interferes with protein analysis (Sun et al., 2008).

Chromatographic separations

Chromatography columns have also been employed in the purification of PPOs. Hydrophobic columns like phenyl sepharose, DEAE, and hydroxylapatile columns are used for the separation of the protein, based on the different size and shape of the accessible hydrophobic regions on their surface (Wissemann and Montgomery, 1985). Kader et al. (1997) found a 19-fold increase in blueberry PPO activity after passage through a phenyl sepharose CL4B column. Similar findings have been reported for mulberry (Arslan et al., 2004) and artichoke (Dogan et al., 2005). For PODs, a DEAE column was used followed by SG-100 column chromatography (Deepa and Arumughan, 2002).

METHODS FOR MEASURING ENZYMATIC ACTIVITY

A number of quantitative methods are available to evaluate PPO and POD activity as listed by Yoruk and Marshall (2003) and shown in Table 3. The chronometric and monometric methods are old and details are difficult to find. The spectrophotometric method measures the rate of quinone formation from *o*-diphenols at 400 - 500 nm, depending on the substrate used. For example, the increase in absorbance at 410 nm with catechol and at 470 nm with guaiacol was measured for PPO and POD activity, respectively (Dann and Deverall, 2000; Yang and Wang, 2008). This method is now the standard technique,and has been used in various studies, including on longan (Jiang 1999), *Protea neriifolia* R Br and *Leucospermum* 'Rachel' (Dai and Paull, 1997). The polarographic method involves measurement of oxygen consumption with an oxygen sensitive electrode. It has been applied in a study of PPO in pear (Richard Forget and Gauillard, 1997). Mayer *et al.* (1966) established that the measured initial apple PPO activity was highest with the polarographic method as compared to the spectrophotometric, chronometric and monometric methods. For diphenolase assays, the polarographic and spectrophotometric methods are rapid,

practical, and considered as more accurate than the other methods (Yoruk and Marshall, 2003).

Table 3. Various methods of measuring PPO activity as a diphenolase assay.

Method	Procedure	Tools
Monometric	Measurement of oxygen consumption over time	Warburg apparatus
Polargraphic	Measurement of oxygen consumption over time	An electrode sensitive to oxygen
Chronametric	Record of the time when colour is first detected	-
Spectrophotometric	Measurement of the formation of o-quinone overtime	Spectrophotometer/ microplate reader

Yoruk and Marshall (2003).

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