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Biochemical properties of purified polyphenol oxidase from bitter leaf (*Vernonia amygdalina*)

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ABSTRACT

Polyphenol oxidase which is responsible for oxidative conversion of phenolic compounds to polymers, has continued to attract the attention of scientists. Here, we report the extraction, purification and biochemical properties of polyphenol oxidase (PPO) from bitter leaf (*Vernonia amygdalina*). The enzyme was purified and concentrated using a non-conventional approach, aqueous two-phase partitioning (ATPS) and the biochemical properties of the purified enzyme were investigated. Substrate specificity studies revealed that the enzyme predominantly exhibits diphenolase activity. The order of substrate preference was catechol > L-DOPA > caffeic acid > L-tyrosine > resorcinol>2-naphthol > phenol. The optimum pH and temperature obtained for the enzyme using catechol as substrate were 5.5 and 50 °C respectively. The estimated Michaelis constant (K_m) and maximum velocity (V_{max}) for the purified *va*PPO using catechol as substrate were 183 ± 5.0 mM and 2000 ± 15 units/mg protein respectively. The catalytic efficiency (V_{max}/K_m) of the purified *va*PPO was 10.9 ± 0.03 min/mg. Na^+, K^+ and Ba^{2+} remarkably activated the enzyme and the level of activation was proportional to the concentration. The *va*PPO presented stability in the presence of up to 50 mM of the different metal ions tested. In contrast, Cu^{2+} and NH_4^+ inhibited the enzyme even 10 mM concentrations. The enzyme was stable in chloroform retaining up to 60% relative activity at 50% (v/v) concentration. There was an increase in the activity (143%) of the enzyme at 30% (v/v) chloroform., revealing that *va*PPO could catalyze the substrate more efficiently in 30% (v/v) chloroform. Total loss of enzyme activity was observed at 20% (v/v) concentrations of acetone, ethanol and methanol.

In conclusion, the properties of the *va*PPO such as its catalysis in the presence of organic solvents, metals and high temperature would be of interest in many biotechnological applications.

1. Introduction

Polyphenol oxidase (PPO) is a metallo-enzyme that contains copper in its active site [1]. The enzyme is known to catalyze oxidation of the hydroxyl group of polyphenols leading to formation of quinones, a reactive compound that led to brown pigments observed in organisms [2]. The oxidation of endogenous phenolic compounds by polyphenol oxidase in plants, fruits and vegetables produces

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melanin and this has always led to reduction in nutritional value, appearance and other organoleptic properties of the plant products [3]. Polyphenol oxidase contains two copper-binding domains at the active site and proteolytic site close to the C-terminus [4]. Polyphenol oxidase is widely distributed in nature and it's essential for several functions in living organisms [5]. The enzyme is found in different tissues and organs, including flowers, leaves, vascular tissue and roots [6]. PPO is important for the biosynthesis of flavonoids, lignin and tannins in plants [4]. PPO has been exploited in various fields such as health, medicine and pharmaceuticals [7]. Due to the potentials of polyphenol oxidase as a catalyst for oxidative conversion of phenolic substrates, it has been deployed for use in various biotechnological applications [8]. These applications include synthesis of cross-linked protein networks [9,10], phenolic waste water detoxification [11], tailoring of polymers [12] and synthesis of pharmaceuticals [13].

Different sources of PPO have reported including bacteria, fungi, animal and plants [14,15]. The PPO found in humans is responsible for skin pigmentation including development of freckles [2]. Plant PPO has an important role in plant stress resistance and physiological metabolism. Most of the PPO in plants are found in the chloroplasts of photosynthetic cells and the leucoplasts of storage cells [16]. PPO has been extracted, purified and characterized in various plants including avocado [17], banana pulp [18], apple fruit [19] and sweet potato [20].

Bitter leaf (*Vernonia amygdalina*) is a valuable medicinal plant that is widespread in West Africa. It is known as bitter leaf due to its characteristic bitter taste and flavour, and can be used as an active anticancer, antibacterial, antimalarial and antiparastic agent [21]. It is a shrub or small tree that can reach 23 feet in height when fully grown with a grey or brown coloured bark, which has a rough, flaked texture [22]. Previous studies have shown that extracts of bitter leaf can boost the immune system and regulate the blood cholesterol level, which is a risk factor for heart attack and stroke [23]. Similarly, the leaf can be consumed to treat fever, feverish condition, joint aches, different levels of intestinal complaints, stomach ache, as well as parasite induced disease such as malaria [24]. The plant extract usually turns brown during and/or after processing. This often affect the quality, shelf life and efficacy of the plant extract for many of these medicinal applications. The browning reactions in bitter leaves suggest the presence of enzymes such as peroxidase and polyphenol oxidase responsible for conversion of polyphenols [25]. Polyphenol oxidase has continued to attract the interest of biotechnologists, biochemists and industries because of its increasing use in several applications [2]. Although mushroom such as button mushroom (*Agaricus biosporus*) has been the traditional source of the enzyme, there is a shift in the sourcing of the enzyme to higher plants. Plants PPOs are an important class of enzyme system found across plant kingdom. They are associated with wide variety of biological, physiological and biotechnological functions [2]. The diversity in the nature of plants PPOs could be reason for their involvement in various functions including plant defense and pigment formation [26]. The large distribution and complexity of genes in most plant PPO necessitates wide diversity in the physical and biochemical properties to perform numerous roles in various plant species under variable conditions [26]. In addition, they are easily attached to insoluble supports to allow operational stability and reusability compared to PPOs from other sources [27,28]. Therefore, the study aimed to establish the presence of PPO in bitter leaf (*Vernonia amygdalina*), purify and document its biochemical properties for use as alternative enzyme in diverse biotechnological applications.

2. Materials and methods

2.1. Materials

Fresh leaves from the stem of a matured *V. amygdalina* were obtained from farms in Owo, Southwestern Nigeria (latitude: 7.1676679 and longitude: 5.5850837). The leaf was authenticated at the Department of Plant Science and Biotechnology, Achiever University, Owo, Ondo State, Nigeria. The accession/voucher number of the plant was not made available and was not deposited.

2.1.1. Chemicals

Catechol, 2-naphthol, 3,4-dihydroxyphenyl-L-alanine (L-DOPA), caffeic acid, resorcinol, L-tyrosine, bovine serum albumin (BSA), Coomassie brilliant blue R-250, phenol, blue dextran, pyrogallol, trizma base, trizma HCl, citric acid, acetic acid, sodium citrate, sodium phosphate dibasic (Na_2HPO_4), anhydrous sodium phosphate monobasic ($NaHPO_4$), copper II chloride ($CuCl_2$), hydrated barium chloride ($BaCl_2.2H_2O$), potassium chloride (KCl) and ammonium chloride (NH_4Cl) were obtained from Sigma Chemical Company, St Louis, USA. Standard markers for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was obtained from Carl Roth GmbH, Karlsruhe, Germany. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Extraction of PPO from bitter leaf

The fresh leaves of *V. amygdalina* were rinsed in distilled water and homogenized in 50 mM sodium phosphate buffer, pH 6.5 on ice to obtain 30% homogenate. The 30% homogenate obtained was subjected to centrifugation at 10,000×*g* for twenty (20) min at 4 °C. After centrifugation, the supernatants were kept in freezer (at −20 °C) for further analyses.

2.2.2. Standard assay for polyphenol oxidase

Polyphenol oxidase activity with catechol as substrate was determined according to the method of Wititsuwannakul et al. [29] as modified by Ilesanmi et al. [30]. The reaction involved final concentration of 5 mM catechol, 50 mM sodium phosphate buffer, pH 6.5 and appropriate volume of enzyme. The rate of formation of benzoquinone was monitored at 410 nm for 2 min at 30 °C using UV-VIS spectrophotometer (Wincom, SHP1002419296) to obtain change in absorbance of 0.02–0.07/min. Possibility of substrate

auto-oxidation was investigated using reaction mixture without the enzyme. This served as the control for the experiment. The PPO activity was thereafter calculated. The experiment was performed in triplicate. One unit of PPO activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of benzoquinone per minute under standard assay condition (ε of benzoquinone = 3400mM-1 cm-1). The PPO activity was calculated using the formula; PPO Activity (μmole/min) = $\frac{\Delta OD_{410nm} \times V \times DF}{\varepsilon \times v}$ where V = total assay volume, DF = dilution factor, Ɛ = extinction coefficient of product, ΔOD = change in absorbance at 410 nm and v = volume of enzyme used.

2.2.3. Protein concentration determination

The concentrations of protein in the crude and purified *va*PPO were determined following the method of Bradford [31] using bovine serum albumin as standard protein.

2.2.4. Purification of crude vaPPO

The crude homogenate of bitter leaf was subjected to aqueous two-phase partitioning (ATPS). The polymers comprised the mixture of PEG6000, ammonium sulphate and sodium chloride at 24, 7 and 2% w/v respectively. The polymer was continuously stirred with appropriate amount of enzyme that makes the mixture 100% at 4 °C. The mixture was left on ice for 6 h in the refrigerator. The mixture phase-separated into two layers. The upper layer contained undesired proteins, unreacted polymers and other contaminants. The upper layer was removed using Pasteur pipette. Both upper and bottom layers were analyzed and assayed for PPO activity. The bottom phase (enzyme-rich) was thereafter separated and stored for further use. The ATPs fraction containing the enzyme was layered on Sephadex G-100 gel filtration column (1.0 × 40 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.5. Forty (40) fractions of 1 mL each was collected at a flow rate of 12 mL/h. Elution was performed with the equilibration buffer and fractions with PPO activity was pooled. Molecular weight of the native enzyme was thereafter estimated on the calibrated Sephadex G-100 by interpolation of the partition coefficient (K_{av}) values of the standard proteins and *va*PPO on the standard curve.

2.2.5. Homogeneity test and determination of subunit molecular weight by sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Enzyme purity and the subunit molecular weight of the purified *va*PPO was determined by SDS-polyacrylamide gel electrophoresis using 12% (w/v) running gel and 2.5% (w/v) preparation (stacking) gel, according to Laemmli [32], modified by Weber and Osborn [33] using buffer system (Tris-glycine) at pH 8.3. Purified PPO (100 μL) in sample buffer (0.12 M Tris base containing 4% (w/v) SDS, 2 M 2-mercaptoethanol, 40% (v/v) glycerol and 0.02% (w/v) bromophenol blue) was boiled for 5 min at 100 °C. Ten microliter (10 μL) of the denatured sample together with the same volume of protein standards: Myosin (212 kDa), β- Galactosidase (118 kDa), BSA (MW 67 kDa), Ovalbumin (MW 45 kDa), Carbonic anhydrase (29 kDa) and Trypsin Inhibitor (20 kDa) ROTI®Mark STANDARD, 0.2 mL, 1 × 0,2 mL Cat No. T851.3 were applied to separate wells on the slab and electrophoresed at 100 V to allow stacking of the proteins. After stacking was achieved, the applied voltage was increased to 150 V to allow separation of the proteins in the resolving gel. After electrophoresis, the protein bands were stained overnight in 1% (w/v) Coomassie brilliant blue R-250 solution. Destaining was performed in a solution containing 10% (v/v) acetic acid and 10% (v/v) methanol in distilled water. After destaining, the length of the gels and distance migrated by various protein bands were measured. The relative mobility (Rm) of each of the protein bands was obtained by making use of the relationship below; Relative mobility = $\frac{\text{Length of the gel before staining} \times \text{Distance of protein migration}}{\text{Length of the gel after staining} \times \text{Distance of the dye migration}}$.

A standard curve was obtained by plotting the molecular weights of standard proteins against their respective relative mobility. The subunit molecular weight of the purified *va*PPO was estimated by interpolation of the relative mobility values obtained from the standard curve.

2.2.6. Substrate specificity

Substrate specificity was carried out following the method of Wititsuwannakul et al. [29]. PPO activity was monitored spectrophotometrically by oxidation of 5 mM final substrate concentrations for L-DOPA at 475 nm (Ɛ = 3600 M^{-1} cm^{-1}); Catechol at 410 nm (Ɛ = 3400 mM^{-1} cm^{-1}) L-tyrosine at 472 nm (Ɛ = 1490 M^{-1} cm^{-1}); caffeic acid at 362 nm (Ɛ = 11.2 mM^{-1} cm^{-1}); resorcinol at 340 nm (Ɛ = 1393 M^{-1} cm^{-1}); 2-naphthol at 475 nm (Ɛ = 6000 M^{-1} cm^{-1}) and phenol at 475 nm (Ɛ = 3300 M^{-1} cm^{-1}) in 50 mM sodium phosphate buffer, pH 6.5. The relative activities were compared with catechol, which was taken to be 100%.

2.2.7. Effect of temperature on vaPPO activity

The effect of temperature on purified *va*PPO activity was investigated. The reaction mixtures containing 5 mM catechol and 50 mM sodium phosphate buffer, pH 6.5 were incubated at different temperatures from 10 to 80 ° C for 5 min. Thereafter, the enzyme was introduced. The polyphenol oxidase activity was assayed as earlier described. The activities obtained were plotted against their respective temperatures.

2.2.8. Effect of pH on the activity of vaPPO

The effect of pH on the *va*PPO activity was experimented using buffer systems with the pH range of 3.0–10.0 at 30 °C. The buffer systems and theirpH range used were as follow: 50 mM citrate buffer, pH 3.0–5.0; 50 mM sodium phosphate buffer, pH 5.5–7.5 and 50 mM glycine-NaOH buffer, pH 9.0–11.0.

2.2.9. Determination of kinetic parameters

The effect of different catechol concentrations on the purified *va*PPO was determined. The kinetic parameters (K_m, V_{max} and V_{max}/

K_m) of the *va*PPO were determined by varying the concentrations of catechol between 1 and 25 mM in the assay buffer. Analysis of data were carried out using non-linear regression software (Graph pad prism 5).

2.2.10. Effect of organic solvents on the activity of vaPPO

Effect of both water-miscible and water immiscible organic solvents on the activity of *va*PPO were investigated. Organic solvents (ethanol, methanol, acetone and chloroform) were introduced into the reaction mixture to achieve 0–80% (v/v) final concentration. The residual activities were measured under standard assay conditions mentioned earlier. The activity of *va*PPO on catechol in 50 mM sodium phosphate buffer, pH 6.5 with the absence of organic solvents was valued as 100%.

2.2.11. Effect of metals on vaPPO

The effect of inorganic salts that are common and known to influence industrial enzymes were investigated. Chlorides of sodium, potassium, copper and barium at concentrations ranging from 0 to 50 mM were used. This was performed by addition of varying concentrations of each metal in the assay mixture. The assay mixture without the salts served as control. The residual activities were expressed as a percentage of the control which was taken as 100%.

2.2.12. Statistical analyses

All experiments were carried out in triplicates and statistical analyses were performed with Microsoft excel package and GraphPad Prism 5.

3. Results and discussion

3.1. vaPPO extraction and purification

The presence and properties of *va*PPO were demonstrated in this work. Extraction procedure of crude *va*PPO in the presence of phosphate buffer resulted in a good yield. The extracted crude *va*PPO was subjected to non-conventional purification, ATPS. The purification scheme was efficient enough to give percentage yield and purification fold of 118% and 5 respectively. One of the advantages of ATPS was the eventual concentration, in addition to purification of the resulting enzyme preparation. The increased percent yield obtained in this step, could be due to differential partitioning of the desired enzyme from endogenous inhibitors, contaminants, non-target proteins to the opposite phases. Another possible reason, could be that the polyethylene glycol used during the purification exerted a conformational change on the active site of the enzyme leading to better accessibility to the substrate. Similar observation has been reported by Adewale and Adekunle [34] for peroxidase from *Cola nitida* purified using ATPS. After being subjected to gel filtration on Sephadex G-100, the final percent yield and purification fold obtained were 76% and 6.5 respectively. The purification summary is shown in Table 1. The purification method was effective in removing unwanted proteins, chlorophyll and other impurities from the desired enzyme to other part. ATPS offers many advantages including low process time, low energy consumption and biocompatible environment to the biomolecule due to the presence of large amounts of water in the extraction system [35]. The polyphenol and contaminant free enzyme solution were sufficiently suitable for further analysis. In the work of Adeseko et al. [36], a purification fold of 6.85 with a recovery of 20% was obtained for polyphenol oxidase from African bush mango after being subjected to three-step purification process.

The native molecular weight estimated on calibrated Sephadex G-100 column was 59.4 $\pm$ 2.0 kDa while that of the subunit molecular weight estimated on SDS-PAGE was 31.0 $\pm$ 1.0 kDa as shown in Fig. 1. This revealed that the bitter leaf PPO is homodimeric. The molecular weight determined for *va*PPO was close to that reported for PPO from the fruit peel of *Irvingia gabonensis* (53.22 kDa) [36]. Partially denaturing PAGE showed several isoforms for PPO from Ataulfo mango with molecular weights of 53, 112, and 144 kDa [37].

3.2. Kinetic parameters of purified vaPPO

Kinetic parameters were estimated from the non-liner regression plot of activity against catechol concentrations. The K_m of *va*PPO for catechol was 183 $\pm$ 5 mM. The V_{max} for the *va*PPO was 2000 $\pm$ 15 units/mg protein. Catalytic power or substrate specificity, V_{max}/K_m was estimated as 10.9 $\pm$ 0.03 min/mg. The high K_m value for catechol revealed low substrate binding, however, it's an indication that the active site of *va*PPO can accommodate more substrate (catechol) conversion to product. It revealed that the enzyme's active site is not easily saturated. Several other researchers also reported high K_m values for PPO from other sources. Deepaa and Wong [38] reported K_m value of 357.1 mM for PPO from *Ipomea batata* using catechol as substrate.

Table 1
Purification summary of the *va*PPO.

Sample (ml)	Volume (units)	Total Activity (mg)	Total Protein (units/mg protein)	Specific Activity Yield	% fold	Purification
Crude	10	12,000	55.0	218	100	1.0
ATPS	6	14,200	13.0	1090	118	5.0
Gel filtration	17	9120	6.4	1417	76	6.5

ATPS – Aqueous two-phase partitioning.

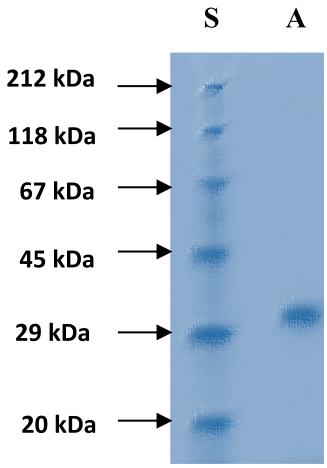

Fig. 1. SDS-PAGE of purified polyphenol oxidase from *V. amygdalina*. S - standard protein markers: Myosin (212 kDa), β-Galactosidase (118 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20 kDa). A-purified polyphenol oxidase from *V. amygdalina* with subunit molecular weight estimated as 31 kDa.

3.3. Temperature effect on the activity of vaPPO

Fig. 2 shows the effect of temperature on purified *va*PPO at temperatures ranging from 10 to 80 °C. The optimal temperature for this *va*PPO is 50 °C. The purified enzyme gradually lost its activity at temperature below 50 °C which could be as a result of less kinetic energy or above 50 °C due to thermal denaturation. Ilesanmi et al. [30] also reported optimum temperature of 50 °C for tyrosinase from different yam species. Polyphenol oxidase from latex of *Hevea brasiliensis* was reported to have optimum temperature of 60 °C [29]. However, an optimum temperature of 30 °C was reported for PPO from sweet potato [38]. The optimum temperature obtained for *va*PPO was 50 °C. While this is in agreement with other reports, some researchershave reported low optimum temperatures for PPO from other species. Wahyudi et al. [39] reported optimum temperature of 35 °C for PPO from potato. In the same vein, PPO from blueberry was at 35 °C [40]. The optimum temperature of the PPO from apple pulp was reported to be 20 °C [41], whereas that of PPO from rambutan peel was at 37 °C [42]. PPO from sorrel showed the highest activity at 30 °C when using catechol as substrate [43]. Optimum temperature of 30 °C was also reported for PPO from peppermint [44], but these temperature values were different from Barbados cherry PPO [1]. Enzymatic activity at high temperature is useful in industrial processes. The variations and discrepancies in optimal temperature of PPOs could be dependent on species, evolutionary trend and habitat temperature.

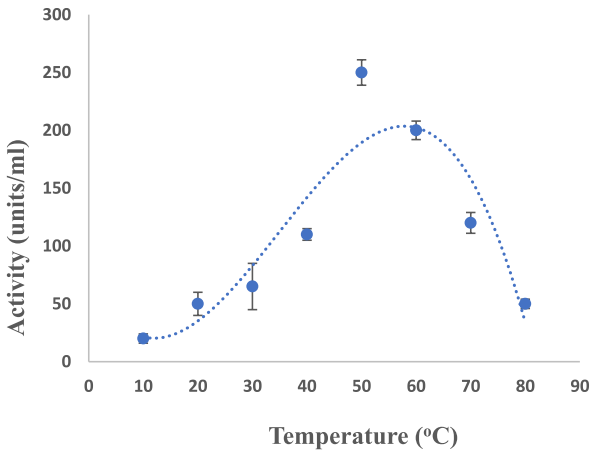

Fig. 2. Effect of temperature on the activity of purified PPO from V. amygdalina. The PPO activity was determined using catechol as substrate at each temperature ranging from 10 to 80 °C under the standard reaction conditions. The activities of the PPO from bitter leaf were plotted against their respective temperature. The optimum temperature obtained was 50 °C. The error bars represent the standard deviation (s.d) of three independent determinations.

3.4. Effect of pH on the purified vaPPO activity

When the activities of *va*PPO were plotted against the pH, the highest activity was found between pH 4.5 and 6.5 (Fig. 3). The optimum activity for *va*PPO was obtained at pH 5.5. Our result was in good agreement with the work of Bravo and Osorio [45] who reported optimum pH of 5.5 for PPO from Cape gooseberry fruit. Daroit et al. [46] reported optimum pH 5.0 for PPO from *Araucaria augustifolia* seeds. An optimal pH of 5.0 was also determined for PPO from Henry chestnuts [47]. Minor variations in reported values can likely be explained by the nature of the enzyme source, the substrate used for activity measurement, and the purity of the enzyme. However, most results confirm that PPO especially from plant origins are not significantly active under basic conditions [2]. pH influences the enzyme activity by affecting the ionization of the prototropic groups in the enzyme's active site. They are possibly involved in maintaining the proper conformation of the active site, in the binding of substrate to the enzyme and/or in transforming the substrate to its products. The optimal pH of the various plant PPOs has been shown to vary from pH 4–8 [47].

3.5. Substrate specificity studies on purified vaPPO

Substrate specificity of the purified *va*PPO is summarized in Table 2. The enzyme had the highest activity at catechol. This was followed closely by L-DOPA. Both catechol and L-DOPA almost gave the same activities with the purified enzyme. Low *va*PPO activities were obtained when caffeic acid and L-tyrosine (monophenol) were used as substrates. Resorcinol, phenol and 2-naphthol were not good substrates for the enzyme probably as a result of the position of their OH groups. Plants PPOs have different enzymatic activity on various phenolic substrates. The position and number of hydroxyl groups have remarkable effect on the PPO's activity [20]. In addition, the specificity of polyphenol oxidase towards substrates are also influenced by the plant cultivar [26]. Field bean PPO was reported to possess high specificity towards 4-methyl catechol and catechol than pyrogallol while in contrast polyphenol oxidase from sunflower seed has higher specificity towards pyrogallol and gallic acid [48].

3.6. Effect of organic solvents on the activity of purified vaPPO

The use of enzymes in organic solvents has continued to attract the attention of biotechnologists and biochemists. We have also reported the activity of *va*PPO in some miscible and immiscible organic solvents. *Va*PPO activity was studied in various organic solvents. Fig. 4 shows the effect of methanol, ethanol, acetone and chloroform on the activity of purified *va*PPO. The enzyme was active in chloroform retaining up to 60% activity at 50% (v/v) concentration. There was an increase in the activity (143%) of the enzyme at 30% (v/v) chloroform revealing that *va*PPO could catalyze better in 30% (v/v) concentration of chloroform. During biocatalysis in non-aqueous media, enzymes are usually active in protic solvents such as DMSO, ethanol, acetone, etc. Than in aprotic ones such as chloroform, ether, hexane, etc. [49]. The *va*PPO was inactivated in all the protic solvents (methanol, ethanol and acetone), but active in chloroform at up to 30% (v/v). Hydrophobic solvents (water-immiscible) are usually better than hydrophilic ones (water-miscible), as the latter have a greater tendency to strip tightly bound water from the enzyme molecule [50]. Water is an integral part of the enzyme and when stripped off, there is a conformational change in the structure thus causing inactivation of the enzyme. An increase in the enzyme activity in presence of 30% (v/v) chloroform, could be due to increased solubility of the substrate/products, resulting in increased bioavailability and potential decrease in potential product inhibition. Biocatalysis in organic solvents also permits advantages such as suppression of many water dependent side reactions, decreased microbial contamination in addition to high selectivity, high specificity and catalytic rates under mild conditions [51]. Shuster and Fishman [52] reported increase in *Bacillus megaterium* tyrosinase activity in the presence of 20% (v/v) DMSO. Similarly, there was increase in yam tyrosinase activity in the presence of 40% ether [30]. Total loss of enzyme activity was observed at 20% (v/v) concentrations of acetone, ethanol and methanol. The potential of polyphenol oxidase as a catalyst in non-aqueous systems could be exploited in green chemistry and organic synthesis.

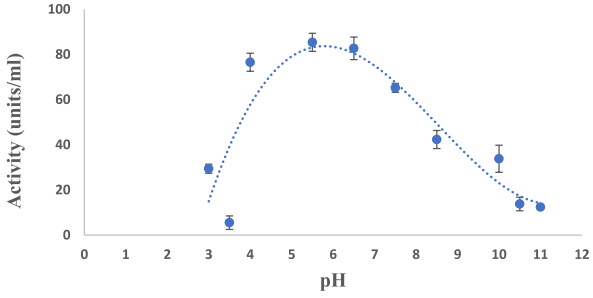

Fig. 3. Effect of pH on the activity of purified PPO from *V. amygdalina*. The *va*PPO was introduced into different buffer system with varying pH and the PPO activity at this pH was determined using catechol as substrate. The activity was plotted against their respective pH. The optimum pH obtained was 5.5. The error bars represent the standard deviation (s.d) of three independent determinations.

Table 2
Substrate specificity of the purified *va*PPO.

Substrates	Relative Activity (%)
Diphenols	
Catechol	100 ± 0
L-DOPA	99 ± 2
Caffeic acid	15 ± 3
Resorcinol	0 ± 0
Monophenols	
L-tyrosine	10 ± 2
2-naphthol	0 ± 0
Phenol	0 ± 0

The data are the mean ± standard deviation (s.d.) of three independent determinations.

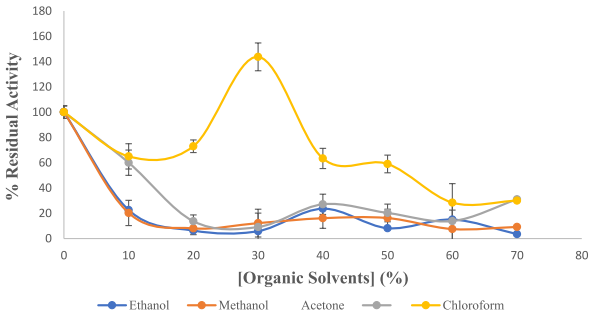

Fig. 4. Effect of organic solvents on the activity of purified PPO from *V. amygdalina*. The plot of % residual activities against different concentration of organic solvents. The activity of the purified PPO in 50 mM phosphate buffer, pH 5.0 at room temperature using catechol as substrate was used as hundred percent (100%) activity. The error bars represent the standard deviation (s.d) of three independent determinations.

3.7. Effect of metals on the activity of purified vaPPO

The influence of metal ions and other compounds on the activity of *va*PPO is usually designed to understand the control strategy of undesirable browning in the plants and plant product processing. These have important applications in foods, cosmetics and drugs industries. The summary of the effect of metals on the activity of *va*PPO is as shown in Table 3. The activity of *va*PPO was effectively inhibited by chloride of copper ions, not retaining up to 40% residual activity even at concentration less than 10 mM. Polyphenol oxidase is a copper-containing enzyme, therefore exposure of additional copper ions into the active site of the enzyme may be inhibitory. This is also in agreement with the report of Uzunoğlu et al. [53] who reported very strong inhibitory effects of copper ions on PPO from banana. Both Hg^{2+} and Sn^{2+} metal ions had maximum inhibitory effect on dill PPO [54]. Kumar et al. [1] also reported inhibition of cherry PPO by copper ions. In contrast, the activity of the *va*PPO was activated by chlorides of potassium, sodium and barium ions to concentration up to 30 mM. Similar activation was observed for sorrel PPO [43]. The activity of PPO from banana was activated in the presence of Fe^{2+} and Ag^{+} [53]. Activation of dill PPO in the presence of Fe2+ was also reported by Arabaci [54]. The activation of vaPPO in the presence of these metals could be that they served as an electrophile, thus stabilizing a negative charge on a reaction intermediate. Another possible reason could be that metal ions bind the substrate, increasing the number of interactions with the enzyme and thus the binding energy. Metals especially heavy metals affect the enzyme activities at cellular level resulting in enzyme activation or inhibition. Some metals are essential for plant growth as they are involved in living processes. However, some metals are considered to be toxic to plant and other living organisms [53].

4. Conclusion

The presence of polyphenol oxidase (PPO) in *V. amygdalina* has been established in this work. The enzyme could thrive under high temperatures. Activity at high temperature demonstrated by the purified *va*PPO may be useful model for proper understanding of its application and could be an advantage for industrial processes. The kinetic characteristics possessed by the purified *va*PPO will result to a more efficient enzyme with improved economic potential in various industrial processes. The enzyme is activated in the presence of some metal ions-potassium, sodium and barium. The *va*PPO activity in non-aqueous systems would be of interest in organic synthesis and several other industrial processes. The overall combination of properties suggests its suitability in several technical and biotechnological applications. The combination of biochemical properties obtained in this study would be of interest and applicability in cosmetics, pharmaceuticals and food industries. Consequently, isolation of PPO from bitter leaf would not only increase the availability of the enzyme for industrial and biotechnological processes, but also add value and improve usability of the plant that is primarily grown for medicinal purposes.

Table 3
Effect of metals on the activity of purified *va*PPO.

Concentration of Metals (mM)	Relative Activity (%)			
	$CuCl_2$	NaCl	KCl	$BaCl_2.2H_2O$
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
10	39 ± 2	101 ± 2	154 ± 6	116 ± 5
20	35 ± 3	143 ± 4	129 ± 2	101 ± 3
30	34 ± 1	120 ± 5	116 ± 3	140 ± 4
40	32 ± 2	79 ± 2	67 ± 3	130 ± 2
50	31 ± 3	91 ± 3	75 ± 2	83 ± 1

The data are the mean ± standard deviation (s.d.) of three independent determinations.

Author contribution statement

Olutosin Samuel Ilesanmi: Conceived and designed the experiments; Wrote the paper.
Omowumi Funke Adedugbe, Oluwaseun Emmanuel Agboola: Contributed reagents, materials, analysis tools or data.
David Adeniran Oyegoke: Analyzed and interpreted the data.
Rachael Folake Adebayo: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

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

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