Biochemical characterization and thermal inactivation of polyphenol oxidase from elephant foot yam (*Amorphophallus paeoniifolius*)

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Abstract Polyphenol oxidase from *Amorphophallus* corm was purified (5.54 fold) by acetone precipitation and ion exchange chromatography. Electrophoretic-blot analysis revealed that the molecular weight of enzyme is 40 kDa. Enzyme preferred catechol as substrate and showed optimum activity at pH 6 and 35 °C with K_m of 17.56 mM and V_{max} 0.023 U/ml/min. Thermal inactivation kinetics study showed that 5.68% inactivation was achieved at 75 °C within 40 min. The half-life time of PPO is between 126.00 and 32.54 min. The activation energy (E_a) and Z values are 64.22 kJ/mol and 34.01 °C, respectively. The inhibitory action of L-ascorbic acid, sodium azide, NaCl, $CaCl_2$, $ZnSO_4$ and EDTA were studied. Thermodynamic studies indicated a non-spontaneous ($\Delta G > 0$) and endothermic reaction process. Other thermodynamic activation parameters, such as E_a , $\Delta H^\ddagger$, $\Delta G^\ddagger$ and $\Delta S^\ddagger$, were also studied. *Amorphophallus paeoniifolius* polyphenol oxidase can find application in baking industry.

Keywords *Amorphophallus paeoniifolius* · Polyphenol oxidase · Thermal inactivation · D and Z value

Introduction

Amorphophallus paeoniifolius, commonly known as elephant foot yam is a tropical tuber crop of Aroid family. Aroids are the oldest cultivated tuber crops rich in carbohydrates, protein, minerals and vitamins. Being the staple food of rural, urban poor of sub-tropics and developing world, it is a source of income for millions of small farmers. The tubers have been used for centuries particularly in traditional medicine for the treatment of asthma, rheumatism, piles, abdominal disorders and tumours. The pharmacological studies showed that corm has analgesic activity, gastro-protective, anti-helminthic, anti-bacterial, anti-convulsing, anti-diarrhoeal, anti-inflammatory, antioxidant activity (Singh and Wadhwa 2014).

Processing of elephant foot yam corm would extend the shelf life and enhance its utilization as crop and also promote food security, meeting nutritional needs as well as increase the revenue margin of farmers. It is well known that unfavourable oxidative browning occurs in the damaged tissue caused during various food processing operations. Polyphenol oxidase (PPO, a copper-containing enzyme) is one of the most common browning agents in nature and this enzyme catalyzes the hydroxylation of monophenols to O-diphenols and oxidation of O-diphenols to O-diquinones. Poly-phenolic compounds also affect to sensory, aroma and nutritional qualities of food. This undesirable enzymatic browning occurs in many plants and vegetables and is of great concern to food technologists and processors. Research on PPO is mainly focused on its role in darkening of damaged tissue along with browning (Mayer 2006).

Browning is also a prominent problem for elephant foot yam derived products. It is known that storage, processing and handling leads to browning of vegetables and limits the

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utilization as has been demonstrated in case of potato tubers (Marri et al. 2003), artichoke (Aydemir 2004; Dogan et al. 2005), Yam (Graham-Acquaah et al. 2014) and lettuce (Gawlik-Dziki et al. 2008) making them less appealing to consumers and thereby resulting in loss of their marketability.

Browning of dough was seen during production of bread that utilized *A. paeoniifolius* flour high in dietary fibre 12.15 g/100 g (Singh and Wadhwa unpublished data) affecting visual attributes of bread. The nutrient content however was not affected as the bread was found to be rich in calcium and dietary fibre (Singh et al. 2015).

The objective of our study was to evaluate the inactivation kinetics and study the thermodynamic parameters of polyphenol oxidase from *A. paeoniifolius* corm. Inhibition study of enzyme by thermal and chemical compounds needs to be addressed so as to minimize the aesthetic losses because of undesirable browning as well as give a theoretical basis to prevent the enzymatic browning during processing. This study would provide the food industry with a new enzyme source thereby increasing potential food processing applications.

Materials and methods

Plant material and chemicals

A. paeoniifolius corms were purchased from local vegetable market at Noida, India. Fresh corm without the peel was processed for phytochemicals and enzyme extraction (Singh et al. 2013, 2014). Fresh corms were rinsed under running water, peeled and chopped into small cubes of 4 mm, rinsed with distilled water and then stored at -20°C till further processing. Pyrocatechol, ascorbic acid, EDTA, D and L Tyrosine were procured from CDH, New Delhi. PVP (Polyvinylpyrrolidone), potassium mono-hydrogen phosphate and potassium di-hydrogen phosphate, SDS (sodium dodecyl sulphate), DTT (dithiothreitol) were procured from Sigma Chemical Co (St. Louis, USA). Macro-prep DEAE support of Biorad was used for purification of enzyme. All reagents used were of analytical grade.

Preparation of the crude polyphenol oxidase (PPO) extract

All the steps of enzyme extraction were carried out at 4°C . Stored cubes of (100 gm) *A. paeoniifolius* corm were homogenized in (chilled pH 7.0, 0.1 M phosphate buffer 1:3 along with 1% of (PVP) polyvinylpyrrolidone). Crude enzyme extract was centrifuged at 10,000 rpm for 30 min at 4°C ; the supernatant was filtered through one layer of

Whatman No.1 and stored at -20°C in small aliquots of 200 μl till further use.

Purification of the PPO extract

Total protein from crude extract was extracted by acetone precipitation at 4°C for 2 h and filtering through Whatman paper No.1, followed by storage at 4°C for 24 h so that the traces of acetone are removed from the acetone precipitate. The acetone free precipitate was re-suspended in 100 mM phosphate buffer (pH 7.0), stirred for 10 min and the suspension was centrifuged at 5000 rpm for 10 min. This suspension was loaded on pre equilibrated (100 mM phosphate buffer pH 8.0) DEAE (Diethylaminoethyl) anion exchanger (Macro-prep DEAE support) column. Unbound of DEAE was collected in fractions of 2 ml each. Elution was done with buffered solution (pH 8.0) containing 10, 20, 50 mM NaCl. Eluate fractions (2 ml) were collected and dialysed to remove the salt. Both unbound and dialysate were assayed for polyphenol oxidase activity. Active enzyme fraction were pooled and stored at -20°C .

Measurement of the enzyme activity

PPO activity was determined spectrophotometrically by measuring the initial rate of quinone formation, which is indicated by an increase in absorbance at 420 nm. The reaction mixture contained 1 ml of 0.1 M phosphate buffer (pH 7), 1 ml of 50 mM catechol solution, 0.1 ml of enzyme solution and 0.9 ml of distilled water. The reaction was carried out for 15 min at 35°C . One unit of PPO activity was defined as the amount of enzyme that caused an increase of 0.001 unit of absorbance per minute under standard assay conditions (Ahmet et al. 1999).

Protein estimation

Protein content was ascertained according to the dye binding method of (Bradford 1976) using bovine serum albumin (BSA) as standard.

Gel diffusion assay for polyphenol oxidase quantification

Agarose (1.5% (w/v); phosphate buffer pH 7.0) was heated in microwave oven and 50 mM catechol was added only after the mixture reached 50°C (Zocca et al. 2008). 25 ml of this buffered solution was transferred into petri dish, cooled to room temperature and stored at 4°C in dark conditions. Crude enzyme extract (20–100 μl) were loaded in 5 mm diameter wells that were made on agarose petri dish. The linearity of the gel diffusion assay was studied by

monitoring the dark ring diameters formed after 12 h of incubation at room temperature.

Detection of PPO activity by electrophoretic-blot technique

Preparation of catechol paper

Dried substrate paper (catechol) was prepared after immersing 3 mm (15 × 12 cm) chromatography paper in 50 mM catechol solution for 10 min followed by drying at 35 °C for 15 min. The substrate paper can be stored in dark condition till further use.

SDS PAGE and electrophoretic-blot

For determining the molecular weight of *A. paeoniifolius* PPO enzyme, 20 µg proteins from crude, acetone and unbound fractions were loaded on 10% SDS PAGE gel. After electrophoresis, one part of gel was stained with Coomassie Blue R-250 for molecular weight determination whereas the other side of the glass plate along with the gel was removed and immediately blotted onto the top of the dried catechol paper (Cheng et al. 2007) for activity staining. A dark activity brown band developing within 5–10 min validates the presence and molecular weight of PPO.

Determination of the enzyme properties

Substrate specificity and kinetics

The reaction system consisted of 1 ml of 0.1 M phosphate buffer pH 7, 0.1 ml of enzyme solution and 0.9 ml (50 mM) of various phenolic compounds as substrate solutions (viz. catechol, gallic acid, L tyrosine, DL tyrosine, pyrogallol, chlorogenic acid and resorcinol). The PPO activity was measured after 10 min of incubation at 35 °C with catechol as reference value (100%).

For determination of Michaelis constant (K_m) and maximum velocity (V_{max}), PPO activities were measured using catechol as substrate at various concentrations ranging from 5 to 125 mM. The reaction velocity [V] was determined by monitoring change in absorbance per min. K_m and V_{max} values of the enzyme were calculated from the slope and intercept of the straight line plot of $1/V$ versus $1/S$ Lineweaver and Burk plot.

Effect of pH and pH stability on PPO activity

The effect of pH on PPO activity was determined under standard assay conditions using 0.1 M hydrochloric acid

potassium chloride buffer (HCl–KCl) (pH 2), 0.1 M citrate buffer (pH 3–6), 0.1 M potassium phosphate buffer (pH 7–8), 0.1 M Tris–HCl buffer (pH 9). The blank contained only substrate solution and reaction buffer. The optimum pH and enzyme activity were expressed as percentage of maximum enzyme activity under standard assay condition.

The pH stability was determined by modified method of Onsa et al. (2000), where in 50 µl of enzyme solution, 1 ml of 0.1 M buffer were incubated at different pH for 24 h at 4 °C and residual activity (%) was determined.

Effect of temperature and thermal stability on PPO activity

Buffered enzyme solution and substrate (50 mM catechol) were separately pre-incubated at different temperature ranging from (35 to 75 °C) for 10 min. Following pre-incubation both the components were mixed and time of incubation was noted. The enzyme and substrate mix were incubated for 10 min at the desired temperature. The reaction was stopped and relative enzyme activity was calculated as the percentage of the highest activity left after temperature treatment.

Thermal stability of enzyme was also determined where buffered enzyme mixture was incubated at the desired temperature 55–75 °C for 5–40 min at desired temperature, in water bath, then rapidly cooled over ice bath so as to reach 35 °C following which the substrate (catechol) solution (held at 35 °C) was added and residual enzyme activity found. The residual enzyme activity is a ratio expressing relative enzyme activity (%) to the original unheated enzyme activity. The first order inactivation constant, (k) was determined from the slope of first order enzyme inactivation equation:

$$\log(\% \text{ residual activity}) = -(k/2.303)t. \quad (1)$$

Half-life ($t_{1/2}$) value of inactivation is given by the expression:

$$t_{1/2} = \ln(2)/k. \quad (2)$$

Results of the thermal experiments were processed into kinetic parameter values, decimal reduction time (D) and its dependence on temperature expressed by Z .

The decimal reduction time was calculated according to (Stumbo 1973) as:

$$D = 2.303/k. \quad (3)$$

The Z parameter was derived from $\log D$ values at different treatment time versus temperature. The Z value indicates how many degrees the temperature increase is required for decimal reduction time to be tenfold higher or lower.

Thermodynamic analysis

Arrhenius' law is usually used to describe the temperature dependence of k -values, and it is algebraically given by

$$\ln(k) = \ln(A) - (E_a/R) * (1/T). \quad (4)$$

The activation energy (E_a) was estimated by slope of the straight line obtained using Arrhenius plot of the logarithm of the rate constant ($\log k$) versus the inverse of absolute temperature.

Where R is the universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), A is the Arrhenius constant, E_a is the activation energy (energy required for the inactivation to occur) and T is the absolute temperature in Kelvin.

Obtained values of the Activation energy (E_a), the activation enthalpy ($\Delta H^\ddagger$) for each temperature was calculated was by

$$\Delta H^\ddagger = E_a - RT. \quad (5)$$

The free energy of inactivation ($\Delta G^\ddagger$) can be determined according to the expression:

$$\Delta G^\ddagger = -R * T * \ln(k * h_p / K_B * T). \quad (6)$$

Activation entropy ($\Delta S^\ddagger$) calculated by

$$\Delta S^\ddagger = \Delta H^\ddagger - \Delta G^\ddagger / \Delta T \quad (7)$$

where K_B is the Boltzmann constant ($1.38 \times 10^{-23} \text{ J/K}$), h_p is the Planck constant ($6.626 \times 10^{-34} \text{ J s}$), and T is the absolute temperature, k (s^{-1}) the inactivation rate constant of each temperature.

Effects of inhibitors

Sodium azide, sodium chloride, ascorbic acid, citric acid, CaCl_2 , ZnSO_4 and EDTA were used as PPO inhibitors. The reaction mixture contained 1 ml of 0.1 M phosphate buffer, pH (7.0), 0.95 ml of 100 mM inhibitor, 0.50 ml of enzyme extract and 1 ml of 50 mM catechol. Percentage inhibition was calculated using the following equation:

$$\text{Inhibition}(\%) = (A_o - A_i / A_o) 100,$$

where A_o : initial PPO activity (without inhibitor) A_i : PPO activity with inhibitor.

Application of crude extract having PPO activity

Dough rising

Salt, sugar, yeast (1 g each) and wheat flour (100 g) were weighed and kneaded in water along with 1 ml of PPO enzyme to form dough whereas no enzyme extract was added to the control dough sample. The dough mixture was transferred to marked beaker and kept at room temperature. The rise in dough was measured at equal time intervals.

Results and discussion

Extraction and partial purification of PPO

Polyphenol oxidase enzyme from corm of *A. paeoniifolius* was extracted in 0.1 M phosphate buffer pH 7 and 1% PVP followed by acetone precipitation and DEAE ion exchange chromatography. Undesirable reaction between the natural phenols and PPO was prevented by adding 1% polyvinylpyrrolidone (PVP). PVP is a phenol adsorbing agent and added to the extraction buffer to remove phenolic substrates due to its binding ability to the phenolics in order to prevent phenol–protein interactions. After acetone precipitation the pellet was suspended in 0.1 M phosphate buffer (pH 7.0) and loaded onto a DEAE-cellulose ion-exchange column. Activity rich DEAE unbound fractions were pooled and dialysed prior to studying its kinetics. DEAE eluate of NaCl 10, 20 and 50 mM did not show any enzyme activity. We report 1.24 fold purification with a yield of 18.92 after acetone precipitation and unbound enzyme fractions of DEAE gave 5.54 fold purification and a yield of 3.19 (see Table 1).

Catechol agarose gel diffusion assay for quantification of PPO

Crude enzyme (20, 40, 50, 60, 100 μl) was added in the wells and kept for 12 h along with (enzyme blank) control (see Fig. 1). Diameters of the oxidized zones were measured and graph was plotted with the log units of the enzymes. The standard curve generated shows linear

Table 1 Purification of PPO from *A. paeoniifolius*

Purification step	Volume	Protein (mg/ml)	Total protein (mg)	Activity (U/ml)	Specific activity (U/mg protein)	Total activity	Yield	Purification fold
Crude extract	50	0.0735	3.68	833	11,333.33	41,650	100	1
Acetone precipitation	4	0.14	0.56	1970	14,071.43	7880	18.92	1.24
Unbound DEAE anion exchange chromatography	2.25	0.0094	0.0212	590	62,765.96	1327.5	3.19	5.54

Fig. 1 Identification and quantitation of PPO activity. Catechol agarose plate assay: 5 mm diameter wells were loaded with crude enzyme (20–100 µl) incubated at 35 °C for 12 h in dark

relationship between the diameters of dark oxidized circles produced in the gel and the logarithm of the units of enzyme activity added to the well. A linear regression analysis revealed a correlation coefficient of 0.8921 (Figure not shown). The test is straightforward and technologically easy. It can be performed in a minimally equipped laboratory, giving very reliable results.

pH optimum and stability

The effect of pH on PPO activity was determined by estimating enzyme activity at different pH 2.0–9.0 using catechol as substrate. *Amorphophallus* PPO shows optimum activity at pH 6.0 (see Fig. 2a). The activity was found to increase up to pH 6.0 after which it dropped considerably giving a bell-shaped curve. Our results are comparable with activities from other plant sources. PPO from peach pulp showed optimum pH around 6.5–7.0 (Liang et al. 2015), pH 7.0 for artichoke and Barbados cherry (Dogan et al. 2005; Kumar et al. 2008). *A. paeoniifolius* corm PPO enzyme activity decreased in extreme alkaline or acidic pH. Enzyme however maintained approx 31% its activity at pH 8. Variation in optimum pH of PPO from different sources are affected by factors such as cultivar, nature of phenolic substrate, type of buffer and ionic strength of buffer, purity of enzyme, extraction method and isoenzyme form (Erat et al. 2010; Palma-Orozco et al. 2011). The pH stability is an important parameter for the determining the conditions that should be available through isolation, purification, handling and storage of the enzyme. Enzyme retained 84% its activity even after 24 h storage at 4 °C at pH 5 whereas only 56% and 20% activity was retained at pH 7, pH 8 respectively (see Fig. 2a). Maximum enzyme activity was found at pH 6. We

conclude that in order to reduce browning reaction the products need to be maintained below pH 5 and above pH 7 before processing.

Temperature optimum and stability

Avoiding optimal temperature would be practical and efficient method to inhibit the enzyme activity and preventing browning reactions in food (Liu et al. 2013). Figure 2b shows the effect of various temperatures on PPO activity. Result shows the optimum temperature of PPO was 35 °C and its activity decreased gradually as temperature increased. Optimum temperatures requirement for PPO of other sources like butter lettuce is reported to be at 35 °C (Gawlik-Dziki et al. 2008), 40 °C for Barbados cherry (Kumar et al. 2008) and 25 °C for artichoke (Aydemir 2004).

The effect of temperature on PPO stability was investigated by incubating the enzyme at higher temperatures in the range of 55–75 °C for 5–40 min. The enzyme retained 5.68% of activity when incubated for 40 min at 75 °C when compared with the activity found at 25 °C which is considered to be 100% (Fig. 2 c). It was observed that the rate of enzyme inactivation (k) increases with increase in temperature. Higher rate constant means the enzyme is less thermo stable at higher temperature (Pal and Khanum 2010). k values for 55 °C was lowest whereas maximum at 75 °C i.e. 0.005 and 0.022 respectively (see Table 2).

The decimal reduction time (D value) is a parameter commonly used in the study of enzyme stability and is defined as the time needed for 90% reduction of the initial activity. D values for *A. paeoniifolius* PPO ranged between 419 and 108 min. The decimal reduction D value, at different temperatures and Z value for temperature (55–75 °C) is presented in Table 2. Stability of enzyme enhances with increase of D value. We report the maximum D value reaches at 55 °C (418.65) min and the minimum D value was at 75 °C (108.10) min. The Z value is characterised by the temperature dependence of the decimal enzyme reduction activity time, which is also defined as the range of temperature increase needed for a $\log_{10}$ reduction in the D value. In general, low Z values indicate greater enzyme sensitivity to heat, and high activation energy (E_a) values reflect a greater sensitivity of the enzyme to temperature change. Z value found in this study was 34.01 °C (Table 2).

The energy of activation E_a is calculated by Arrhenius plot. The activation energy E_a for *Amorphophallus* PPO was $E_a = 64.22$ kJ/mol ($y = -7724.1x + 18.307$, $r^2 = 0.9942$).

Some of the reported E_a values for PPO include 37.9 kJ/mol for Marula fruit and 87.8 kJ/mol for taro PPO (Mdluli 2005; Yemenicioglu et al. 1999). We can state that

Fig. 2 Determination of enzyme properties (a) Optimal pH requirement as well as effect on stability of enzyme on incubation for 24 h at different pH. (b) Optimal temperature requirement. (c) Effect of incubation time and temperature on the inactivation of enzyme.

Table 2 Thermal inactivation parameters (k , $t_{1/2}$, Z , D) for *A. paeoniifolius* PPO at respective temperature

Temp (°C)	k (min ⁻¹)	R^2	$t_{1/2}$ (min)	D (min)
55	0.005	0.859	126.00	418.65
65	0.011	0.9339	68.61	227.98
75	0.022	0.935	32.54	108.10
Z (55–75 °C) = 34.01				

(d) Effect of inhibitors (100 mM) on enzyme activity respectively. (e) Enzyme kinetics study; The Lineweaver–Burk plot of enzyme activity measured using most preferred catechol as substrate. K_m and V_{max} values are 17.56 mM and 0.023 U/ml/min

A. paeoniifolius PPO is not very sensitive to heat and also not showing sensitivity to temperature changes.

An investigation of other thermodynamics parameters viz; the enthalpy of activation ($\Delta H^\ddagger$), Gibbs free energy ($\Delta G^\ddagger$), and entropy of activation ($\Delta S^\ddagger$), activation energy (E_a) is necessary to understand further the complex process of deactivation study and the behaviour of molecules in different physiological conditions.

Table 3 Thermodynamic parameters of heat inactivation of *A. paeoniifolius* PPO

Ea (KJ/mol)	T (K)	$\Delta H^\#$ (KJ/mol)	$\Delta G^\#$ (KJ/mol)	$\Delta S^\#$ (J/mol K)
64.22	328	61.49	105.94	−135.52
	338	61.41	107.55	−136.51
	348	61.32	108.68	−136.09

Thermodynamic parameters for PPO for *A. paeoniifolius* PPO enzyme were calculated and depicted in Table 3. The numerical values of thermodynamic properties are affected by two major factors, namely solvent effect due to presence of surrounding water molecules and structural effect due to conformational changes occurring in the enzyme molecule during reaction. Our results show that enthalpy has increased in the range of 61.49–61.32 kJ mol^{−1} with increase in temperature from 55 and 75 °C. The Gibbs free energy ($\Delta G^\#$) measures the spontaneity of a reaction. The Gibbs free energy increases with rise in temperature, with its maximum value at 75 °C i.e. 108.68 kJ mol^{−1} presenting that the enzyme has exhibited the resistance against thermal unfolding at higher temperatures. At all studied temperatures, entropy changes were found to be negative. The entropy of the system was found to be −136.09 J mol^{−1} K^{−1} at 75 °C. Negative entropy or negligible disorderliness indicates that PPO enzyme is thermodynamically stable. This is attributed to the formation of charged particles around the compacted enzyme which leads to ordering of solvent molecules (Gohel and Naseby 2007).

Substrate specificity

The enzyme affinity varied depending on the substrate used. PPO specificities were examined in the presence of different substrates. PPO showed substrate preference for di-phenols. Enzyme preferred catechol (considered as 100%), over chlorogenic acid (51.55%) and showed least preference for gallic acid (5.78%), pyrogallol (5.36%) and (below 1%) activity for L, DL tyrosine where as with resorcinol no activity was detected (result not shown).

Kinetic characteristics of PPO

Michaelis–Menten curve of PPO activity towards increasing concentrations of substrate (catechol) is depicted in Fig. 2e. Km value reflects of the affinity of the enzyme towards substrate, smaller values shows greater affinity for its substrate. While, Vmax is rate constant for the formation of product from the enzyme substrate (ES) complex. The Km and Vmax values of PPO calculated from Lineweaver–Burk plot ($y = 763.35x + 43.08$, $R^2 = 0.974$) were found to be 17.56 mM and 0.023 U/ml/min, respectively.

Fig. 3 Detection of PPO activity by SDS PAGE (a); electrophoretic-blot technique (b). **a** Single dark brown band was clearly visible in all the 3 lanes (C, Crude enzyme extract; A, Acetone precipitated fraction; U, unbound fraction obtained after DEAE ion exchanger) depicts that the molecular weight of *A. paeoniifolius* polyphenol oxidase is 40 kDa. The native gel was kept on dry catechol paper along with the glassplate and dark brown activity bands appeared after 5 min of contact

Km value (17.56 mM) was comparable to that of Banana PPO 18 mM (Galeazzi and Sgarbieri 1981), Persimmon fruit of 12.4 mM (Özen et al. 2004) and Mulberry 19.81 mM (Arslan et al. 2004).

Molecular weight determination of *Amorphophallus* corm PPO

SDS PAGE and the semi native SDS PAGE (protein sample was diluted with sample buffer devoid of β -mercaptoethanol and loaded without heat treatment) revealed that the approximate molecular weight of PPO to be 40 kDa. SDS PAGE (Fig. 3a) and the electrophoretic blot (Fig. 3b) where the native gel was kept on dry catechol paper along with the glassplate for 5 min showed presence of single dark brown band, clearly visible in all the 3 lanes instantly. These bands confirm the presence of PPO in all the three fractions (C; crude enzyme extract, A; acetone precipitated fraction, U; unbound fraction of DEAE).

Molecular weight of the enzyme can also be estimated very accurately by this blot zymography studies.

Enzymes isolated from various plant sources suggested that the PPO enzyme can exist as monomer as reported in *Musa paradisiaca* Leaf and *Lactarius piperatus* PPO 40 kDa (Diwakar and Mishra 2011; Oz et al. 2013), Butter lettuce 60 kDa (Gawlik-Dziki et al. 2008) and Wheat bran 37 kDa (Yoshiki et al. 2008).

The dimeric form of the enzyme is reported by (Fang et al. 2007) in Pawpaw fruit where it has been shown to have two isoforms with MWs of 28.2 and 38.3 kDa and by (Sun et al. 2012) in rape flower of 60.4 kDa.

Electrophoretic blot analysis of *A. paeoniifolius* PPO enzyme showed only one activity band around 40 kDa proving the monomeric nature of the enzyme and the band also appeared almost at the same position as in SDS-PAGE (Fig. 3 a, b).

Effect of inhibitors on PPO activity

Use of sulfites and its substitutes have been restricted by U.S. FDA because of adverse health issues. Alternative measures such as pressure infiltration of inhibitors into the cut fruit and vegetables, chemical permeabilization, blanching need to be looked into and methods standardized for controlling enzymatic browning. There are a number of inhibitors, such as sodium metabisulphite, L-cysteine, sodium azide, tannic acid, benzoic acid ascorbic acid used by the researchers to prevent enzymatic browning (Yang et al. 2001). The effect of seven inhibitors—L-ascorbic acid, sodium azide, NaCl, CaCl_2 , ZnSO_4 , EDTA, citric acid on *A. paeoniifolius* PPO are shown in Fig. 2d. L-ascorbic is the most preferred inhibitor of *A. paeoniifolius* PPO. Ascorbate acts as an antioxidant also by reducing the initial quinone formed by the enzyme to the original diphenol before it undergoes secondary reactions which lead to browning.

Application in dough rising

Dough raising capability of the crude extract of *A. paeoniifolius* was monitored at regular intervals. Maximum dough rising takes about 8 h after which there was no increase in height of the risen dough. Crude enzyme treated dough, showed better dough rising property with a difference of 0.8 cm between control, and enzyme treated dough (Fig. 4).

Previous studies have reported that PPO affects the dough by affixing dough-enhancement additives to the bread dough, exert an oxidizing effect to the dough, strengthening gluten bonds, increasing volume, reducing stickiness which results in improved freshness of the bread texture, flavour and the improved machinability resulting in improved crumb structure and softness of the baked

Fig. 4 Application of crude PPO for dough rising in bakery. (a) indicates control (without addition of PPO) (b) and (c) shows the PPO treated dough samples with 1 ml in duplicate. Bars indicate the dough-raising in control and PPO treated dough samples (0.8 cm more rise in height of dough)

product (Minussi et al. 2002). Role of crosslinking of esterified ferulic acid on the arabinoxylan portion of the dough also brings about softening phenomenon as there is breakdown of the cross-linked arabinoxylan network (Cabrero et al. 2007). *A. paeoniifolius* PPO may be affecting the different phenolics existing in dough and its association with biomolecules in flour can be accounted for the observed changes which need to be investigated further.

Conclusion

Enzymatic browning causes considerable economic and nutritional loss in the commercial production of fruits and vegetables products. *A. paeoniifolius* PPO is not sensitive to temperature changes and is a very stable enzyme. It has half-life time between 126.00 and 32.54 min. The D (108–418 min) Z (34.01 °C), k (0.0055–0.0213) values and the high values obtained for activation energy indicates that high amount of energy is needed to initiate denaturation of PPO. The enzyme is however sensitive to L ascorbic acid and citric acid and pH change. Food products from *A. paeoniifolius* where inhibition of PPO is desired the production pH should be below pH 5 and above pH 7.0. We propose alternative strategies to prevent enzymatic browning in *A. paeoniifolius* by using combination of temperature, pH and chemical permeabilization with ascorbic acid, sodium chloride. *A. paeoniifolius* PPO enzyme will find application in dough rising and baking industry where bond strengthening is required, this property of enzyme stability at high temperature would be economical.

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Compliance with ethical standards

Conflict of interest Authors have no conflict of interest.

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