Partial purification and characterization of the quality deteriorating enzymes from Indian pink guava (*Psidium guajava* L.), var. *Lalit*

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Abstract Peroxidase (POD), polyphenol oxidase (PPO), ascorbate oxidase (AO) and pectin methylesterase (PME) from ‘Lalit’ guava variety have been purified using BioGel P-100 and UNOsphere Q resins. POD, PPO, AO, and PME were partially purified to 28, 30, 36 and 30 fold; single band on SDS-PAGE represented a molecular weight of 21, 70, 75, 50 kDa, respectively. Optimum pH for POD and AO was 6.5 whereas, PPO and PME showed optimum pH in the alkaline range of pH 8.0 and 8.5, respectively. AO showed optimum activity at 25 °C, POD and PPO showed optimum activity at 35 °C; however, PME showed maximum activity at 55 °C. AO was the most heat labile whereas, PME was the most heat stable enzyme studied and may carry an important role in the thermal processing of guava pulp as an indicator of the efficacy of treatment.

Keywords *Psidium guajava* · Peroxidase · Polyphenoloxidase · Ascorbate oxidase · Pectin methyl esterase · Anion-exchange chromatography

Introduction

Enzymes play an essential role in the development of fruits, also during their processing and storage. Fruit contains many endogenous enzymes some of which are beneficial for developing sensorial properties while others result in deteriorating fruit quality characteristics such as

color, flavor, texture, and nutritional value. Endogenous quality deteriorative enzymes such as POD, PPO, PME, and AO can add upon the loss of quality of fruit products. POD and PPO are oxidoreductases involved in enzymatic browning process, bringing about the reduction of diphenols to *o*-quinones which are then polymerized to undesirable brown, red, or black pigments (Chisari et al. 2007). PME which has been found in plants, as also in pathogenic fungi and bacteria, catalyzes the hydrolysis of methyl ester groups of pectin and is responsible for cloud loss in juices and nectars (Ly-Nguyen et al. 2002). AO is a copper-containing enzyme, which catalyzes the aerobic oxidation of L-ascorbic acid (Tokuyama et al. 1965).

Though these enzymes are present in most of the plants, their expressivity would vary depending on the species, age, and stage of maturation (Vámos-Vigyázó and Haard 2009; Vishwasrao and Ananthanarayan 2016a, b). To improve the quality, storage, and processing characteristics of the fruits, it is necessary to understand the biochemistry of these enzymes from this fruit source while plenty of literature is available for purification and characterization of these enzymes from various other plant sources (Mondal et al. 2009; Sener and Umit Unal 2011; Rojas-Reyes et al. 2014).

Earlier reports suggest that enzyme characteristic changes with source which makes it necessary to study enzymes from various sources. Though *Lalit* variety of pink guava is a well-known fruit and has a great market potential (Vishwasrao and Ananthanarayan 2016b); very scanty information is available about the enzymes and their biochemistry. Therefore, the present study deals with extraction and partial purification of POD, PPO, PME, and AO from *Lalit* variety of pink guava. Further, the characterization of partially purified enzymes by determining

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kinetic parameters, optimum conditions of pH and temperature, pH and thermal stability have been studied.

Materials and methods

Materials

Fully ripe *Lalit guava* (*Psidium guajava* L.) fruits (Skin brightness, L value = 60.81 ± 4.18 ; $a^* = 3.30 \pm 0.47$) were harvested from *guava* orchard located in Pune, India and brought to the research laboratory in cardboard boxes (at temperature of 25 °C). Bovine serum albumin-V, hydrogen peroxide, triton X-100, ammonium sulfate were procured from Himedia laboratories Pvt. Ltd., India. Polyvinylpyrrolidone (PVP), sodium chloride (NaCl), guaiacol, pectin, phenol red, L-ascorbic acid and catechol were purchased from SD Fine Chemicals Pvt Ltd., India. BioGel P-100, UNOsphere™ Q, and Bradford reagent were obtained from Bio-Rad, US. All chemicals used in the analysis were of analytical grade.

Methods

POD, PPO and AO extraction and purification

The flow diagram (Fig. 1) depicts the extraction and purification process in brief. The fruits after seed removal were crushed using a blender to obtain a pulp. Phosphate buffer (200 mM) of pH 7.0 containing 2 mM EDTA, 0.3% PVP, 1 M NaCl and 1% triton $\times$ 100 was prepared (Ortuño et al. 2013). Pulp (1:2, w/v) was then stirred in the buffer for 90 min in ice bath. The solution was then centrifuged (Beckman Coulter J2-MC, Brea, US) at $12,064 \times g$ at 4 °C for 30 min. The supernatant was filtered through filter paper (Whatman #1) using vacuum filtration and filtrate was used as crude enzyme extract for estimation of POD, PPO and AO activities. Further, crude enzyme extract was mixed with solid ammonium sulphate (saturation of 0–80%) for precipitation of the proteins and allowed to stand for 8 h at 4 °C. The precipitate was collected by centrifugation (Beckman Coulter J2-MC, Brea, US) at $12,064 \times g$ for 30 min at 4 °C and re-dissolved 0.05 M phosphate buffer of pH 7.0. This was then dialyzed overnight against four changes (4×1 L) of the buffer at refrigerated conditions, centrifuged and later applied to a column packed with BioGel® P-100 (protein concentration was 290 $\mu\text{g/mL}$). BioGel® P-100 has 45–90 μm wet bead size and fractionation range of 5–100 kDa. The (10 cm $\times$ 1.5 cm) column, with a 17.68 mL packed volume, was equilibrated and eluted with 30 mM potassium phosphate buffer of pH 7.0 using BioLogic DuoFlow™ medium-pressure chromatography systems (Bio-Rad, Hercules, CA,

US). Total of 13 fractions of 4 mL size were collected throughout the run with a flow rate of 6 mL/h and each fraction was monitored for the protein amount (280 nm) as well as for the enzymatic activities. Two millilitre (protein concentration was 30 and 20 $\mu\text{g/mL}$ for POD and PPO/AO, respectively) of fraction obtained from size exclusion chromatography using BioGel® P-100 which gave the highest activity of POD, PPO and AO was loaded on to UNOsphere™ Q anion exchange column (9 cm $\times$ 1.5 cm, 15.91 mL) which was equilibrated with Tris–HCl Buffer (30 mM, pH 8.5). UNOsphere™ Q resin is a strong anion exchanger which has 120 μm bead size and pH stability for pH range 2–14. Elution was carried out with the same buffer containing NaCl (0–200 mM). Total of 32 fractions of 3 mL size were collected with a flow rate of 60 mL/h and was monitored for the protein amount (280 nm) as well as for the enzyme activities (manually using enzyme assay). Fractions obtained from various stages of purification showing enzyme activity were loaded on a SDS-PAGE gel to check its purity and to determine the molecular weight of the enzymes. The partially purified enzyme fractions were then used to characterize pH and temperature optima, pH and temperature stability and substrate dependency.

PME extraction and purification

Pulp obtained from blending of fresh fruit after removal of seeds was mixed with DW (1:1, w/v) and stirred for 30 min, solution was then centrifuged (Beckman Coulter J2-MC, Brea, US) at $12,064 \times g$ at 4 °C for 30 min. Sediment thus obtained was re-extracted in 8.8% NaCl solution (4:15, w/v) of pH 7.5 for 120 min and then centrifuged (Beckman Coulter J2-MC, Brea, US) at $12,064 \times g$ at 4 °C for 30 min. The supernatant was filtered (Whatman #1) using vacuum filtration and filtrate was used as crude enzyme extract for estimation of PME activity (Vishwasrao and Ananthanarayan 2016a). Crude PME extract was further treated with solid ammonium sulfate (saturation of 0–30%) for precipitation of the proteins. The precipitate obtained by centrifugation (Beckman Coulter J2-MC, Brea, US) at $12,064 \times g$ for 30 min at 4 °C was discarded; the supernatant was further saturated with solid ammonium sulfate (saturation of 30–80%) and the precipitate was collected. The precipitate was then re-dissolved 0.03 M phosphate buffer of pH 7.5 and dialyzed overnight against four changes (4×1 L) of the same buffer at refrigerated conditions. The solution was then centrifuged (Beckman Coulter J2-MC, Brea, US) at $12,064 \times g$ for 30 min at 4 °C and 1 mL (protein concentration was 240 $\mu\text{g/mL}$) of clear supernatant was later applied to a column packed (9 cm $\times$ 1.5 cm, 15.91 mL) with UNOsphere™ Q anion exchange resin. The column was equilibrated with Tris–

Fig. 1 Flow diagram representing the steps involved in extraction and purification of POD, PPO and AO from guava fruit

HCl Buffer (30 mM, pH 8.5). Elution was carried out with the same buffer containing NaCl (0–200 mM). Total 37 fractions of 3 mL size were collected with a flow rate of 60 mL/h and was monitored for the protein amount (280 nm) using BioLogic DuoFlow™ medium-pressure chromatography systems (Bio-Rad, Hercules, CA, US) as well as for the enzyme activities (manually using enzyme assay). Fractions obtained from various stages of purification showing enzyme activity were then loaded on a SDS-PAGE gel to check its purity and to determine the molecular weight of the enzymes. Further, this partially purified PME fraction was used to study the effect of pH, temperature, and substrate on enzyme activity.

Protein determination

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

Assay for determination of enzyme activity

Phosphate buffer was prepared in DW obtaining a molar concentration of 0.05 M and pH of 7.0. Mixture of hydrogen peroxide (30 μ M) and guaiacol (40 mM) in phosphate buffer was used as a substrate solution for estimating POD activity after incubated at 25 °C for 15 min (Ciou et al. 2011). PPO activity was estimated using 20 mM catechol in 0.03 M potassium phosphate buffer of pH of 6.5, incubated at 30 °C for 15 min before using it in the assay (Aydin and Kadioglu 2001). AAO activity was measured using a reaction mixture composed of 500 μ L phosphate buffer (200 mM, pH 6.5) containing 0.5 μ M L-ascorbic acid and 100 μ L of the enzyme. After 10 min, the reaction was stopped by the addition of 1.48 mL of 0.2 M HCl, and the absorbance was measured in a spectrophotometer at 245 nm. The control was prepared with the inactivated extract by adding 0.2 M HCl before addition of the substrate (Gomez and Lajolo 2008). One unit activity of POD, PPO and AO was defined as change in absorbance of 0.001 units (at 475 and 420 nm, respectively) per minute (Vishwasrao and Ananthanarayan 2016b). PME activity was determined using 0.5% pectin as substrate and 0.01% phenol red solution (0.003 mol/L potassium phosphate buffer) and adjusting the pH of the final solution to 7.5 (Hagerman and Austin 1986). The substrate mixture was incubated at 25 °C for 15 min before usage. One unit activity of PME was defined as change in absorbance of 0.001 units (at 545 nm) per minute.

Determination of molecular weight

The molecular weight of the purified enzyme was estimated by SDS-PAGE. The separating gel contained 12% polyacrylamide (29:1, acrylamide: bisacrylamide), 375 mM Tris pH 8.8, 0.1% SDS, and 0.1% ammonium persulfate. The stacking gel contained 5% polyacrylamide (29:1, acrylamide: bisacrylamide), 125 mM Tris pH 6.8, 0.1% SDS, and 0.1% ammonium persulfate. The gel was run in Bio-Rad mini PROTEAN 3 cell gel apparatus at a constant voltage (100 V) at 24 °C for 4 h in Tris–glycine electrophoretic buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.1% SDS). Midrange-3 protein marker (Sisco Research Laboratory Pvt Ltd, India) was run along with the purified fractions to estimate the molecular weight of the enzymes. Protein bands were visualized by subjecting the gel to silver staining.

pH activity profile and pH stability

POD, PPO and PME activity of the partially purified enzyme, as a function of pH, was determined in a pH range of 3.0–5.5 in 50 mM acetate buffer, 6.0–7.5 in 50 mM phosphate buffer and 8.0–9.5 in 50 mM Tris–HCl buffer. The pH stability of the partially purified enzyme obtained from final step of purification (ion exchange chromatography) was determined by incubating 0.5 mL of enzyme solution in 0.5 mL of respective buffer solution for 1 h at 24 °C. Residual activities were determined for POD, PPO, AO and PME as mentioned in “[Assay for determination of enzyme activity](#)” section.

Temperature activity profile, thermal stability and heat inactivation

POD, PPO, AO and PME activity of the partially purified enzyme obtained from final step of purification (ion exchange chromatography), as a function of temperature, was determined in a temperature range of 5–95 °C. The temperature stability was determined by incubating 1 mL of enzyme solution at a certain temperature (5–95 °C) in a water bath for 5 min. Residual activities were determined for POD, PPO, AO and PME as mentioned in “[Assay for determination of enzyme activity](#)” section.

Enzyme kinetics

The Michaelis–Menten constant (K_m), the maximum velocity of the reaction (V_{max}), and specificity for the substrate (V_{max}/K_m) were determined by plotting the activity data as a function of substrate concentration according to the method of Lineweaver and Burk (Lineweaver and Burk 1934). Guaiacol (4–100 mM), catechol

(6–500 mM), ascorbic acid (5–100 μ M) and pectin (1–10 mg/mL) were used as substrate for estimating enzyme kinetic parameters of POD, PPO, AO and PME, respectively.

Statistical analysis

All determinations were conducted in at least triplicates. Analysis of variance (ANOVA) and Duncan's multiple range test was employed to determine the statistical significance of the differences between the means ($p \leq 0.05$) by using IBM SPSS statistics 20.

Results and discussion

Extraction and purification of POD, PPO and AO

The steps involved in the purification of POD, PPO and AO enzymes has been summarized in Table 1.

Isolation of enzymes from guava matrix needed a higher salt concentration and was extracted in the presence of anionic detergent. Preliminary steps were performed in

order to set conditions for combined extraction of enzymes; Triton X-100 and PVP were added to extraction buffer to absorb phenolic compounds and to enhance oxidoreductases activity (Rojas-Reyes et al. 2014). Fractional precipitation of crude extract showed that 80% saturation of ammonium sulfate resulted in highest activity recovery for all the three enzymes. The excess of salt was removed by dialyzing the precipitate. The precipitate after dialysis had the specific activity of 49,101.15, 607.22 and 10.12 U mg^{-1} protein, which gave the fold purity of 2.53, 2.59 and 2.46, for POD, PPO and AO, respectively (Table 1). However, the major loss of enzyme occurred at the stage of ammonium sulfate precipitation and can be related to the long standing time (at 5–10 $^{\circ}\text{C}$) followed during precipitation of enzymes. Further, 1 mL of dialysate was separated on BioGel P-100 size exclusion resin; the results are presented in Fig. 2a.

Two major fractions of protein were eluted as fraction no. 3 and 6. PPO and AO activity was found to be present in fraction no. 3, suggesting similar molecular weight of both the enzymes. POD was eluted in fraction no. 6, indicating that the molecular weight of POD to be less than that of PPO and AO. The specific activity of the enzymes

Table 1 Steps involved in partial purification of POD, PPO, AO and PME from *Lalit guava* fruit

	Actual volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purity	Enzyme yield (%)
Purification of POD						
Crude	10.36	2.57	49,881.70	19,433.02	1.00	100.00
0–80% Ammonium sulphate precipitation	1.00	0.29	14,123.80	49,101.15	2.53	28.31
BioGel P-100 (fraction 6)	2.00	0.03	5849.90	184,163.52	9.48	11.73
Unosphere-Q (fraction 12)	1.00	0.01	2907.00	549,100.00	28.26	5.83
Purification of PPO						
Crude	10.36	2.57	601.17	234.20	1.00	100.00
0–80% Ammonium sulphate precipitation	1.00	0.29	174.67	607.22	2.59	29.05
BioGel P-100 (fraction 3)	2.00	0.02	74.66	3917.35	16.73	12.42
Unosphere-Q (fraction 7)	1.00	0.01	33.00	7012.50	29.94	5.49
Purification of AO						
Crude	10.36	2.57	10.57	4.12	1.00	100.00
0–80% Ammonium sulphate precipitation	1.00	0.29	2.91	10.12	2.46	27.52
BioGel P-100 (fraction 3)	2.00	0.02	1.86	97.59	23.69	17.59
Unosphere-Q (fraction 12)	1.00	0.01	0.70	148.75	36.12	6.62
Purification of PME						
Crude	9.76	2.26	517.07	228.68	1.00	100.00
30–80% Ammonium sulphate precipitation	1.00	0.24	72.00	300.00	1.31	13.92
Unosphere-Q (fraction 15)	1.00	0.01	64.00	6800.00	29.74	12.38

Data represents Mean of three independent experiments

Fig. 2 **a** Elution profile of the *guava* POD, PPO and AO in BioGel P-100. **b** Elution profile of *guava* POD in UNOsphere Q anion exchange resin. **c** Elution profile of *guava* PPO and AO in

UNOsphere Q anion exchange resin. **d** Elution profile of *guava* PME in UNOsphere Q anion exchange resin

further intensified after size exclusion chromatography; POD, PPO, and AO had fold purity of 9.48, 16.73 and 23.69, respectively as compared to crude extract (Table 1). Enzyme yield, on the other hand, decreased further with 11.73, 12.42 and 17.59% residual activity obtained in the fractions. The relative higher fold purity of PPO and AO can be related to the lesser quantity of other protein molecules of similar molecular size present in the fraction number 3. Two milliliters of fraction no. 6 containing POD from BioGel P-100 resin was then loaded on UNOsphere Q anion resin, which further purified POD (28.26 fold purity). POD was eluted as a major fraction at 33 mM concentration of sodium chloride in a linear gradient of 0–40 mM. The results obtained are depicted in Fig. 2b. Literature available about purification of *guava* POD is very scanty and no direct comparison with earlier data is possible. However, POD from other plant sources such as avocado, sweet gourd, marula fruit and *Carica papaya* was purified to 14.5, 10.2, 19.0 and 30.2 folds by the use of various chromatography techniques (Mdluli 2005; Koksai et al. 2012; Pandey et al. 2012; Rojas-Reyes et al. 2014).

Similarly, 1 mL of fraction no. 3 obtained from BioGel P-100 resin containing PPO and AO was also loaded on UNOsphere Q anion resin, which further resolved proteins

in multiple fractions. The elution profile obtained has been provided in Fig. 2c. PPO and AO were eluted at 8 and 35 mM concentration of sodium chloride in a linear gradient of 0–40 mM (Fig. 2c). Fraction no. 7 and 12 showed the highest specific activity of PPO and AO (7012.50 and 148.75 U mg⁻¹ of protein) and presented a fold purity of 29.94 and 36.12, respectively as indicated in Table 1. These partially purified enzyme fractions were then studied for determination of enzyme characteristics. Various researchers have studied purification of PPO and AO from different sources using ion-exchange chromatography and/or size-exclusion chromatography and have reported a fold purification of 43, 58 and 76.7 for artichoke PPO, marula PPO and *Averrhoa carambola* fruit AO, respectively (bin Saari et al. 1999; Aydemir 2004; Mdluli 2005).

Extraction and purification of PME

PME being a membrane-bound enzyme, it needs higher salt concentration for isolation from *guava* pulp. The pulp was initially extracted with DW to remove soluble proteins and any present inhibitor. The residue thus obtained was then extracted with 8.8% NaCl solution, the extract thus obtained was considered as crude PME. The steps involved

in the purification of PME enzyme of guava has been summarized in Table 1. Crude extract of PME was further treated with solid ammonium sulfate for fractional precipitation of proteins. The precipitate obtained after saturating crude extract with 30–80% of ammonium sulfate gave the highest activity recovery for PME. This fraction after dialysis showed a specific activity of 300.00 U mg^{-1} of protein with 1.31 fold purification. One milliliter of this dialysate was loaded on UNOsphere Q anion exchange resin and was eluted with increasing salt gradient. The elution profile of guava PME in UNOsphere Q resin has been depicted in Fig. 2d. Proteins were eluted as many fractions at different salt concentration, however, major activity recovery of PME was detected in fraction no. 15, at a salt concentration of 33 mM. The fraction thus obtained showed 29.74 fold purification (Table 1) and was used for enzyme characterization. Earlier research related to guava PME purification from *Paluma* variety reports a very high fold purification (104 fold) using Sephadex G100 size exclusion chromatography (da Silva Cerqueira Leite et al. 2006). Similarly, 129.11 fold purification was reported for guava PME using DEAE-cellulose anion exchange and Sephadex G-100 size exclusion chromatography (Mondal et al. 2009). Moderate fold purification in the present study obtained was related to limited availability of different resins.

Molecular weight determination with SDS-PAGE

Enzyme fractions recovered at various stages of purification such as ammonium sulphate precipitation, size exclusion chromatography and UNOsphere Q anion exchange chromatography were loaded (25 μL fractions containing enzyme activities) onto SDS-PAGE for molecular weight determination. SDS-PAGE stained with silver staining is presented in Fig. 3.

All enzyme containing fractions (Lane 1 in Fig. 3a and Lanes 1, 4, 5 in Fig. 3b) obtained after final purification step of passing through UNOsphere Q anion exchange resin showed a single fraction in denaturing SDS-PAGE indicating that enzymes have been purified to near homogeneity. Partially purified POD, PPO, AO and PME fraction showed a molecular weight of $\sim 21, 70, 75, 50 \text{ kDa}$, respectively. There are no direct reports for the molecular weight of POD extracted from guava fruits, however, a similar range of molecular weight was observed in the case of peach POD (29 kDa) and sapota POD (20 kDa) (Neves 2002; Vishwasrao et al. 2017). AO can exist in monomeric, dimeric or tetrameric forms and exhibit huge variation in molecular weights; literature reports 80 kDa monomeric structure of AO extracted from *Acremonium fungus* (Hirose et al. 1994) and 68 kDa structure in zucchini squash (Hirose et al. 1994) and 68 kDa structure in zucchini

squash (Lin and Varner 1991). Amino acid sequencing of *Cucumis sativus* presented 62.3 kDa structure of AO, which was eventually found to be present in 3 different molecular forms of monomer (30 kDa), dimer (60 kDa) or tetramer (120 kDa) (Esaka et al. 1990).

The molecular weight of guava PPO ($\sim 70 \text{ kDa}$) was similar to wounding-induced PPO from cowpea (*Vigna unguiculata*) seedlings (73 kDa), however, guava infected with fruit rot disease was found to have a molecular weight of 180 kDa (Razzaque et al. 2000; Pinto et al. 2008). In nature, various other PPO comparable to guava PPO includes 76 kDa for litchi fruit PPO, 72 kDa for Indian tea leaf PPO, 70 kDa for plantain PPO and 69 kDa for potato PPO (Mdluli 2005).

Partially purified guava PME obtained after anion exchange chromatography showed a molecular weight of 50 kDa in denaturing SDS-PAGE. Two isoforms of PME have been reported earlier in guava with a molecular weight of 57 and 99 kDa (Carvalho et al. 2009). Other reported PMEs such as kiwi PME and papaya PME showed the molecular weight of 50 and 53 kDa, respectively (Lourenco and Catutani 1984; Ciardiello et al. 2008).

Effect of pH on enzyme activity

The pH of a solution can have several effects on the structure and activity of enzymes. pH can have an effect on the state of ionization of acidic or basic amino acids. State of ionization of amino acids in a protein alters the shape of protein. This can lead to altered protein recognition or enzyme inactivation. The activity of enzymes varies with a change in pH of the substrate solution.

The optimum pH for activity of guava POD and AO was found to be 6.5 with guaiacol and ascorbic acid as substrate (Fig. 4a), respectively. However, optimum pH for activity of guava PME and PPO was observed in the alkaline range (8.5 and 8.0, respectively). Optimum pH for guava POD and AO has not been reported in the literature; however, optimum pH for guava PPO infected with the fruit rot disease and guava PME has been reported to be 8 and 8–9, respectively (Razzaque et al. 2000; da Silva Cerqueira Leite et al. 2006). POD from other fruit sources such as papaya and sweet gourd reported having optimum activity at neutral pH (7.0 and 7.2, respectively) (Koksal et al. 2012; Pandey et al. 2012). However, optimum activity at acidic condition (pH 4.6) has also been reported for avocado POD with guaiacol substrate (Rojas-Reyes et al. 2014). Partially purified AO from *Cucumis melo* and cucumber showed an optimum activity at pH of 5.5 and 6.0 (Sekiya et al. 1990; Moser and Kanellis 1994).

Studies for pH stability suggested that the guava POD was highly stable in the acidic pH range (3.0–5.0) and retained almost 91–99% residual activity after incubation

Fig. 3 Denaturing SDS-PAGE (12% gel) of enzyme fractions obtained at various stages of purification **a** guava POD, **b** guava PPO, AO and PME, stained with silver staining. **a** Lane 1: fraction number 12 from UNOsphere Q column (POD), Lane 2: molecular mass marker, Lane 9: fraction number 6 from BioGel P-100 column;

b Lane 1: fraction number 15 from UNOsphere Q column (PME), Lane 2: dialyzed PME fraction, Lane 4: fraction number 7 from UNOsphere Q column (PPO), Lane 5: fraction number 12 from UNOsphere Q column (AO), Lane 6: molecular mass marker

Fig. 4 Effect of **a** change in pH on optimum activity of POD, PPO, AO and PME, **b** change in pH on stability of POD, PPO, AO and PME, **c** change in temperature on optimum activity of POD, PPO, AO and PME, **d** change in temperature on stability of POD, PPO, AO and PME

for 60 min at 24 °C. Stability of POD decreased slightly in neutral-alkaline pH range (7.0–9.5), with a decrease in residual activity of 10–20% (Fig. 4b). Similarly, PPO from guava was highly stable in the acidic pH range of 3.0–6.0 and residual activity decreased up to 40% at pH conditions of 7.5–9.5 after incubation at 24 °C for 60 min (Fig. 4b). Partially purified AO was stable in near neutral conditions

(6.0–7.0) and was very sensitive to highly acidic or alkaline conditions. On the other hand, PME was highly stable in alkaline condition and its activity was slightly affected by acidic conditions. Guava enzyme presented varying stability towards pH change, POD and PPO was stable in acidic range of pH, AO showed good stability at neutral

pH, whereas PME was found to be stable in alkaline conditions.

Effect of temperature on enzyme activity

Like a chemical reaction, rate of an enzyme catalysed reaction depends upon the reaction temperature. At increased temperature the reaction speed increases as molecules move faster increasing frequency of collisions. However, with continuous rise in temperature further to optimum temperature would result in protein denaturation causing enzyme inactivation and reduction of reaction rate. Temperature along with pH can affect the enzyme activity to a greater extent. The activity of enzyme is highly temperature dependant and the sensitivity of enzyme to temperature can vary with source of enzyme.

The study showed that the optimum temperature for activity of both POD and PPO was 35 °C (Fig. 4c). Though POD and PPO were active in the temperature range of 5–95 °C, at extreme temperatures their activities were curtailed. PPO purified from guava infected with fruit rot disease was found to have optimum activity at a temperature of 48 °C (Razzaque et al. 2000). The optimum temperature for enzyme activity varies with species, cultivar and with the environmental conditions at which fruit has been grown. POD extracted from papaya and sweet gourd have been reported to be optimally active at 40 and 50 °C, respectively (Koksal et al. 2012; Pandey et al. 2012).

AO showed a gradual increase in activity with an increase in temperature and optimum activity was observed at 25 °C. Further increase in temperature resulted in a decrease in the activity of AO, which may be due to the instability of enzyme or its substrate (ascorbic acid) at higher temperatures. About 95% decrease in AO activity was observed at 65 °C and beyond this temperature, enzyme failed to function. Literature does not provide any direct reference to compare the guava AO temperature dependency, though AO extracted from *Cucumis melo* showed an optimum activity at 37 °C (Moser and Kanellis 1994). Out of the enzymes studied, partially purified PME was optimally active at a higher temperature of 55 °C and was not functional at 95 °C. PME purified from Brazilian guava variety showed an optimum activity at 75 °C (da Silva Cerqueira Leite et al. 2006).

Partially purified POD and PPO, when stored at temperatures from 5 to 35 °C, showed good stability. Further increase in temperature resulted in decreased activity of enzymes, POD failed to function when incubated for 10 min at temperatures beyond 65 °C. However, PPO continued to function at 75 °C and showed no activity at temperatures beyond 75 °C when incubated for 10 min (Fig. 4d). AO was an unstable enzyme, and its activity decreased rapidly with increase in the temperature. AO

failed to react when the partially purified enzyme was incubated at temperatures beyond 65 °C for 10 min. PME showed a greater stability towards increasing temperature and displayed 14% residual activity when stored at 85 °C for 10 min.

Enzyme kinetic analysis

Enzyme kinetics study the effect on the reaction rate of an enzyme catalysed reaction as affected by varying reaction conditions of substrate, pH and temperature. We have understood the optimum temperature and pH conditions for enzyme activities in previous section. This section deals with identifying enzyme kinetics related to varying substrate concentration and type of reaction mechanisms followed by the enzymes. The Lineweaver–Burk plots showing Michaelis–Menten type kinetics of POD, PPO, AO and PME have been presented in Fig. 5a–d.

POD and PPO exhibited a typical hyperbolic velocity saturation curve and showed to followed Michaelis–Menten kinetics. K_m and V_{max} values for all the four enzymes were calculated from the Lineweaver–Burk double-reciprocal plots (Fig. 5a, b). K_m and V_{max} values for guava POD were found to be and 19.77 mM, 769.23 U/mL, and 45.38 mM, 384.62 U/mL for guaiacol and H_2O_2 substrates, respectively at optimum conditions (35 °C, pH 6.5). Various POD extracted from fruits reported showing K_m value of 0.8 mM (papaya), 17.1 mM (sweet gourd) with guaiacol substrate (Koksal et al. 2012; Pandey et al. 2012). The ratio V_{max}/K_m of 38.91 (guaiacol) and 8.47 (H_2O_2) indicated a preferential action of the enzyme for guaiacol than H_2O_2 . Partially purified PPO showed K_m and V_{max} of 32.50 mM and 454.55 U mL⁻¹ with catechol as a substrate at optimum conditions of pH and temperature (35 °C, pH 8.0). PPO from guava infected with fruit rot disease showed K_m and V_{max} of 4.4 mM with catechol substrate (Razzaque et al. 2000). A wide range of K_m has been reported using catechol as the substrate; PPO extracted from Stanley plum showed K_m of 20 mM, whereas PPO extracted from Chinese cabbage showed K_m as high as 682.5 mM (Siddiq et al. 1992; Nagai and Suzuki 2001).

Partially purified AO showed K_m and V_{max} value to be 133.00 μ M and 3333 U/mL with AA as a substrate (Fig. 5c). Lower K_m value of 50 μ M has been reported for AO extracted from *Reticulatus* variety of melon (Moser and Kanellis 1994). The K_m and V_{max} of guava PME were measured with aqueous solutions of pectin in the range of 1–10 mg/mL without adding any salt to the substrate. PME also exhibited a hyperbolic velocity saturation curve with K_m value of 5.76 mg/mL and V_{max} of 49.21 U/mL (Fig. 5d) of pectin revealing that PME from guava follows Michaelis–Menten kinetics. PME isoenzymes isolated from *Paluma* variety of guava showed a

Fig. 5 The Lineweaver–Burk plots showing Michaelis–Menten type kinetics of **a** POD using guaiacol as substrate, **b** PPO using catechol as substrate, **c** AO using ascorbic acid as substrate and **d** PME using pectin as substrate

lower K_m of 0.23–0.32 mg/mL (da Silva Cerqueira Leite et al. 2006), another study of guava PME reports K_m of 3.1 mg/mL (Mondal et al. 2009).

Conclusion

Quality deteriorating enzyme viz. POD, PPO, AO, and PME from ‘Lalit’ guava variety have been purified using BioGel P-100 and UNOsphere Q resins. POD, PPO, AO, and PME were partially purified to 28, 30, 36 and 30 fold; single band on SDS-PAGE represented a molecular weight of 21, 70, 75, 50 kDa, respectively. Optimum pH for POD and AO was similar (6.5) whereas, PPO and PME showed optimum pH in the alkaline range of pH 8.0 and 8.5, respectively. AO showed optimum activity at 25 °C, POD and PPO shared optimum temperature of 35 °C; however, PME showed maximum activity at 55 °C. AO was the most heat labile whereas, PME was the most heat stable enzyme studied and may carry an important role in the thermal processing of guava pulp as an indicator of the efficacy of treatment. Further, it was observed that all the enzymes followed Michaelis–Menten kinetics. The Michaelis–Menten constant of POD, PPO, AO and PME with guaiacol, catechol, ascorbic acid, and pectin as substrate was found to be 19.77 mM, 32.50 mM, 133.00 μ M and 5.76 mg/mL, respectively.

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