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Characterization of polyphenol oxidase in ginger (*Zingiber officinale* R.)

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Abstract

A polyphenol oxidase (PPO) isoform that showed expression at all developmental stages of rhizomes in 13 ginger (*Zingiber officinale* R.) accessions and the only one observed at full maturity of rhizome was characterized. The isoform is a non-covalent homo-dimeric protein of 66 kDa subunits. The native molecular mass was estimated at ~127 kDa using non- reducing SDS–PAGE (10%). Its activity after purification was confirmed by substrate staining both in native gel (7%) and non-reducing SDS gel (10%). The N-terminal amino acid sequence of the subunit of ginger rhizome polyphenol oxidase is 'Glu-Gln-Gly-Val-Gly-Gly-Asp-Asp-Gly-Leu-.' The enzyme showed maximum activity at pH 4.5 and 60°C. The PPO is thermo-tolerant and active in a broad pH range (pH 3.5 to 8). Heat inactivation studies showed a decrease in enzyme activity at 75°C and above. Lower concentrations of $MgCl_2$ (1 mM) and $CaCl_2$ (0.5 mM) activated the enzyme whereas higher concentrations (10 mM) reduced the activity. L- Cysteine HCl, L-ascorbic acid, potassium metabisulfite and NaCl inhibited PPO strongly. Western blot analysis of crude leaf extracts with polyclonal antiserum raised against purified PPO confirmed absence of its expression in leaves at different stages of development. Polyclonal anti-PPO antiserum cross reacted with *Solanum tuberosum*, *Raphanus sativus* and *Dioscorea esculenta* tuber extracts and *Solanum melongena*, *Malus sylvestris* and *Musa paradisiaca* fruit extracts but no cross reactivity was observed with *Curcuma amada* and *Ipomoea batatas* extracts.

Keywords: characterization, ginger rhizome, polyphenol oxidase

Abbreviations: PPO=Polyphenol oxidase; PS=Phenyl sepharose; PVP=polyvinyl pyrrolidone; RP-HPLC=Reverse phase-high performance liquid chromatography; SDS-PAGE=Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Introduction

Polyphenol oxidases (PPO) (EC 1.14.18.1 and EC 1.10.3.1) are a group of copper proteins that play a significant role in crop defence against pests and pathogens (Li & Steffens 2002), in dormancy of tubers and sprouting (Marri *et al.* 2003) and also in physiology of fruit ripening (Aydin & Kadioglu 2001). Though PPO from

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various sources including many tropical tuber crops (Adamson & Abigor 1980; Yemenicioglu *et al*. 1999) has been studied, no information is available on the characterization of a PPO from the family *Zingiberaceae* to which ginger (*Zingiber officinale* R.) belongs. In the present work, a PPO isoform, the only one expressed dominantly in fully matured rhizome was isolated and characterized.

Materials and methods

Source of plant materials

Seed rhizomes of 13 ginger accessions namely, IC 276773, IC 276775, IC 130169, IC 84985, IC 136603, IC 136617, IC 248814, IC 248816, IC 248824, IC 432613, IC 432644, IC 280758 and IC 280760 were obtained from National Bureau of Plant Genetic Resources (Regional Station), Thrissur (Kerala). Plants were raised from these seed rhizomes and enzyme activity was assayed from seed rhizomes at two, four and eight months of age. Enzyme was purified from mature rhizome only.

Enzyme extraction

Sodium phosphate buffer, 0.1 M (pH 6.5) containing 10 mM mercaptoethanol and 0.1% PVP was used as extraction buffer. The enzyme extracts from different ginger accessions were prepared separately by homogenizing rhizome (2 g each) in extraction buffer (2 ml) at 4°C. The homogenate was centrifuged at 10000 g at 4°C for 15 min and the supernatant was used as enzyme source. For purification of enzyme, rhizome (100 g fr. wt) was cut into thin slices, homogenized in ice-cold extraction buffer (100 ml) in a mixer grinder for 3 min. The homogenate was filtered through glass wool and the filtrate centrifuged at 10000 g for 30 min at 4°C.

Enzyme assay

PPO activity was determined spectrophotometrically at 400 nm using 4-methyl catechol as substrate. One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmol of 4-methyl-o-benzoquinone per min calculated using a molar extinction coefficient of 1350 M^{-1} cm^{-1} (Ferrer *et al*. 1988). Catalase was used in the reaction mixture to rule out the possibility of peroxidase activity. All PPO assays were performed in triplicate and means reported.

Protein estimation

Protein concentration was estimated following the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Enzyme purification

Proteins in the crude extract of ginger rhizome were precipitated using ammonium sulfate at 60% saturation, re-dissolved in 0.1 M Na-phosphate buffer and equilibrated with 1.5 M ammonium sulfate in the same buffer. The supernatant, after removal of precipitated protein by centrifugation, was subjected to further fractionation through phenyl-Sepharose column. Thirty fractions were collected using gradient elution (1.5 M to 0 M ammonium sulfate in 100 mM phosphate buffer). The absorbance at 280 nm and PPO activity were monitored for each fraction. Fractions with appreciable PPO activity were pooled, dialyzed and concentrated by lyophilization and then loaded onto a DEAE-cellulose column. Fifteen fractions were collected and the absorbance measured at 280 nm and PPO activity assayed. The purity and activity of two consecutive fractions from DEAE-cellulose column which showed the highest specific activity was ascertained by native PAGE, in-gel substrate staining and analytical RP-HPLC.

Polyacrylamide gel electrophoresis

PAGE under native, reducing and non-reducing conditions were performed. For reducing PAGE, the protein sample was diluted with the sample buffer containing SDS 2.5% (w/v), reducing agent, β-mercaptoethanol 5% (v), glycerol 5% (v) and Tris-HCl 62.5 mM (pH 6.8) and heated for 5 min at 95°C. For non-reducing SDS-PAGE, the protein sample was diluted with sample buffer devoid of β-mercaptoethanol and loaded without heating. After the electrophoresis, the gel was stained with Coomassie brilliant blue for visualizing polypeptides.

In-gel staining of PPO activity

Unboiled crude extracts from all 13 accessions were applied without reducing agent to native polyacrylamide gels (7%) and also to SDS-polyacrylamide gels (12% and 10%). Purified PPO (10 µl) unboiled and without reducing agent was loaded to 10% SDS-PAGE and elecrophoresed. In both native and non-reducing SDS-polyacrylamide gel, staining for PPO activity was done by equilibrating the gel in 0.1% p-phenylenediamine in 0.05 M sodium phosphate buffer (pH 6.5) for 30 min followed by immersion in 10 mM catechol in the same buffer.

Analytical RP-HPLC

The DEAE-cellulose column-purified fractions were subjected to RP-HPLC on a Phenomenex Luna ODS 18 column using HPLC (Shimadzu model LC-8A) equipped with a variable length UV detector (190–300 nm) and a microprocessor. Twenty µl of DEAE-cellulose column purified fraction was injected and eluted using 60% acetonitrile in double distilled water containing 0.1% trifluoro acetic acid. The flow rate was 1 ml min^{-1}.

N-Terminal amino acid sequence analysis

SDS-PAGE (10%) was cast and the gel, including stacker was allowed to stand for 72 hours at 37°C prior to use. Thioglycolate, 0.1 mM (11.4 mg l^{-1} of tank buffer) was used in the electrode buffer filled in the upper chamber of the electrophoresis unit to scavenge reactive compounds left in the gel. The purified polyphenol oxidase (0.5 mg) was suspended in double distilled water (200 µl) and mixed with 50 µl of 5X working sample buffer. The sample was incubated at 37°C for 15 min before loading. After electrophoresis, proteins were transferred to PVDF membrane using semi-dry blotting apparatus (Bio-Rad, USA). The N-terminal amino acid sequence was determined on a Shimadzu model PPSQ-21 protein sequenator using a Wako pack-PTH amino acid column (C18) maintained at 40°C. The coupling and conversion reactions were carried out at 45°C and 65°C respectively.

Western blotting and immunostaining

Antiserum was elicited in New Zealand white male rabbit by injecting the purified polyphenol oxidase. Immunization, collection and separation of antiserum were conducted following the methods of Harlow & Lane (1988). The antigen excess antibody capture enzyme linked immunosorbent assay (ELISA) technique of Engvall & Perlmann (1972) was followed for determination of titre of antiserum raised against polyphenol oxidase. The polypeptides after SDS-PAGE were transferred to nitrocellulose membrane according to Towbin *et al*. (1979) using semi dry blotting apparatus (BioRad, USA). Alkaline phosphatase conjugated monoclonal antirabbit IgG (Sigma) diluted (1:5000) in conjugate buffer was used as secondary antibody and 5-Bromo 4-chloro 3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT) (Bangalore Genei, India) as substrate for western blot analysis.

Cross reactivity of ginger PPO antiserum with other crops

ELISA technique was employed for cross reactivity studies. Crude protein extracts from different crops namely apple (*Malus sylvestris* Borkh.), elephant foot yam (*Amorphophallus paeoniifolius* Dennst.), banana (*Musa paradisiaca* L.), brinjal (*Solanum melongena* L.), colocasia (*Colocasia esculenta* L.), coleus (*Solenostemon rotundifolius* Poir.), mango ginger (*Curcuma amada* Roxb.), potato (*Solanum tuberosum* L.), sweet potato (*Ipomoea batatas* L.) and yam (*Dioscorea esculenta* L.) which show high degree of browning while processing were prepared using sodium phosphate buffer (pH 6.8), centrifuged, supernatant collected and their protein contents were estimated (Lowry 1951). The supernatant (200 ml each) was used as antigen. A dilution of 1:4000 of ginger PPO antiserum was used as primary antibody. Alkaline phosphatase conjugated monoclonal antirabbit IgG (Sigma) diluted (1:5000) in conjugate buffer was used as secondary antibody and p-Nitrophenyl phosphate, (pNPP, Sigma Co., USA) as substrate for ELISA and color intensity measured at 405 nm. Crude extracts substituted by buffer were used as control.

Effect of temperature on o-Diphenolase activity and stability

In order to find out the effect of temperature on PPO activity, the purified enzyme and also the reaction mixture without enzyme extract were incubated separately for 15 min at temperatures ranging from 30°C to 75°C prior to measurement of its activity. Buffered substrate without enzyme served as control. PPO activity was measured as described earlier. Heat stability studies were carried out in the temperature range of 30°C–90°C. To eppendorf tubes (2 ml capacity) containing 1 ml of 0.05 M sodium phosphate buffer (pH 6.5), 2 ml of purified PPO was added and incubated at specified temperature for 45 min. All aliquots were then cooled rapidly to 30°C and assayed for residual PPO activity.

Effect of pH on o-Diphenolase activity and stability

Enzyme activity was assayed at varying pH using 4-methyl catechol as substrate. The buffers (50 mM) between pH 2.5 and 12 were used; pH 2.5 to pH 6.0, citrate-phosphate buffer; pH 6.5 to pH 8.5, Na-phosphate buffer and pH 9.0 to 12, carbonate buffer. Different aliquots of partially purified PPO fraction from matured rhizomes were incubated for 24 h at 4°C, at varying pH from 2.5-12. After 24 h, they were brought back to 6.5 and assayed for the residual PPO activity.

Effect of thiols and reducing agents

The PPO activity was measured using the purified PPO in the presence of different inhibitors at 25°C. Different inhibitors such as cysteine HCl, SO_2, NaCl and ascorbic acid at varying concentrations (0.5 mM-20 mM final concentration in the reaction mixture) were prepared in 0.05 M Na-phosphate buffer at pH 6.5. Inhibitor solution (2 ml) was then mixed with 0.5 ml purified PPO and incubated for 30 min at 30°C. Residual activity was measured as described earlier.

Results and discussion

Polyphenol oxidase activity and expression pattern were investigated in relation to the developmental stages prior to its isolation and characterization. In rhizomes of different ginger accessions studied, three isoforms, though varied in level of expression were detected in native PAGE during early and middle stages of rhizome development, whereas a single, predominantly expressed, common isoform was observed at harvest stage. Purified PPO isoform from matured ginger rhizomes was proved to be the same as that of highest molecular mass observed in developing rhizomes (Fig. 1B). Its molecular mass is similar to that of one leaf isoform (Fig. 1B), but native gel (Fig. 1A) reveals that it is unique and expressed in rhizomes and not in leaves. In leaves, fast migrating isoforms were predominantly expressed whereas in rhizomes, slow migrating isoforms showed higher expression levels in comparison to the fast migrating isoform (Fig. 1A).

In tropical climate, where the crop is popularly grown, maturity always coincides with onset of summer when high soil temperature and moisture tension prevails. Dominant expression of PPO of the highest molecular

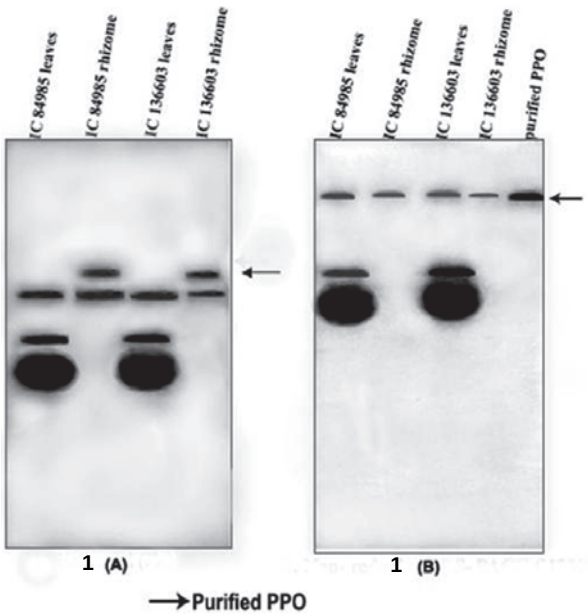

Fig 1. PPO isozymes in leaves and developing rhizomes of ginger accessions. A. Native gel (7%) shows one rhizome specific isoform at cathodic end, B. Non-reducing SDS-PAGE (12%) of same extracts shows that the purified isoform from mature rhizome is of same molecular mass as that of one leaf isoform.

mass in fully matured rhizomes when death of aerial parts occurs and the rhizome becoming dormant is suggestive of some specific physiological functions for this particular isoform associated with either dormancy or stress tolerance.

Purification of polyphenol oxidase

Polyphenol oxidase from the mature ginger rhizome was isolated by ammonium sulfate fractionation followed by PS and anion-exchange chromatography. Ammonium sulfate fractionation recovered 62.2% of the PPO activity. Ammonium sulfate (1.5 M) equilibration of 0-60% ammonium sulfate precipitated fraction resulted in significant loss of PPO activity but lead to further concentration of PPO. Attempts to purify PPO on PS column led to considerable recovery of activity (26.7%) and the proteins resolved in three to four peaks (Fig. 2). No inactive protein peaks were observed but a few fractions with low protein contents that emerged towards the end of gradient elution from PS column always showed high specific activity.

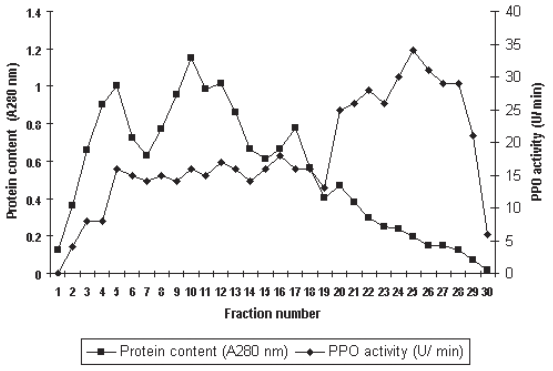

Fig 2. Protein content and PPO activity of phenyl sepharose column fractions.

DEAE-cellulose chromatography of PS column derived PPO activity fractions, on gradient elution with 0-0.2 M ammonium sulfate in 50 mM phosphate buffer, led to purification of PPO by which a homogenous preparation of PPO (1.6 mg protein from 100 g rhizome) with a specific activity of 181.94 U was obtained. Volumes of 5 ml or less were applied to the DEAE cellulose column to obtain maximum resolution. Polyphenol oxidase eluted from the column in two peaks and major active fraction emerged during the second peak and showed the highest specific activity. Almost pure PPO was obtained from phenyl Sepharose column towards the end of gradient elution. The purity of two consecutive fractions with high specific activity eluted in the second protein peak of DEAE chromatogram was ascertained by native PAGE and analytical RP-HPLC and activity confirmed by in-gel substrate staining. The purification steps, purification fold and percentage recovery are shown in Table 1.

The specific activity of the enzyme improved considerably at every stage of purification. As reported in many PPO purification procedures (Partington & Bolwell 1996; Yang et al. 2001; Wang & Constable 2003; Marri et al. 2003), hydrophobic column chromatography

Table 1. Summary of purification of polyphenol oxidase from ginger rhizome

Purification step	Volume (ml)	Total activity *(U)	Total protein (mg)	Specific activity (U $mg^{-1)}$	Percentage recovery	Purification fold
Crude extract	106	2755.9	805.6	3.42	100	1
Ammonium sulfate fractionation	32	1713.7	102.4	16.74	62.2	4.9
Phenyl sepharose column	11	735.8	18.4	39.99	26.7	11.7
DEAE- Cellulose column	10	291.1	1.6	181.94	10.6	53.2

*One unit of enzyme activity is defined as the amount of enzyme that produces 1 ìmol of 4-methyl-o-benzoquinone min^{-1}.

considerably increased the degree of PPO purification (11.7 fold). The PPO isoform purified from mature rhizome in comparison with isoforms from the developing rhizomes and leaves of a few accessions is shown in figure 1B. Among various isoforms detected, purified one is of highest molecular mass (Fig. 1B).

Molecular mass and subunit association

Purified PPO migrated to a position corresponding to that of a protein with a molecular mass of about 66 kDa on SDS-PAGE under reducing conditions (Fig. 3A and 3B), whereas it migrated as a ~127 kDa protein under non-reducing conditions in presence of SDS (Fig. 3C, lane 1 and 5) suggesting its dimeric nature. Molecular mass of native PPO was determined following the method of Wang & Constable (2003) using non-reducing 10% SDS-PAGE and its activity confirmed by substrate staining (Fig. 4C, lane 5). Its subunit association is not through a disulfide linkage as established by the appearance of 66 kDa band in the lane loaded with sample heated in the absence of the reducing agent (Fig. 4C, lane 4). Plant PPOs of similar subunits as observed in the present case (Marri et al. 2003) and also of dissimilar subunits (Partington & Bolwell 1996) were reported earlier.

N-Terminal sequencing of purified polyphenol oxidase

The N-terminal first ten amino acids sequence of PPO from ginger rhizome is Glu-Gln-Gly-Val-Gly-Gly-Asp-Asp-Gly-Leu- (EQGVGGDDGL; P85026). The protein sequence data reported here is available in the UniProt knowledgebase under the accession number P85026. The N-terminal sequence is devoid of sulfur amino acid and is rich in glycine and acidic amino acids. BLAST search did not reveal any consensus sequences with reported PPOs. N- terminal sequence of PPO shows consensus generally among the same species/genus. No PPO sequence information is currently available in zingiberaceae family.

Western blot analysis

SDS-PAGE of purified PPO from mature rhizome and crude leaf extract and crude potato extract were blotted onto nitrocellulose membrane and reacted with PPO antiserum. Secondary labeling of the blots with goat anti-rabbit IgG conjugated with alkaline phosphatase and subsequent substrate staining revealed a 66 kDa protein in lane loaded with purified PPO whereas no band appeared in lanes loaded with crude leaf extracts of different growth stages confirming its rhizome specific expression (Fig. 4). Native gel pattern of leaf and rhizome crude extracts from different

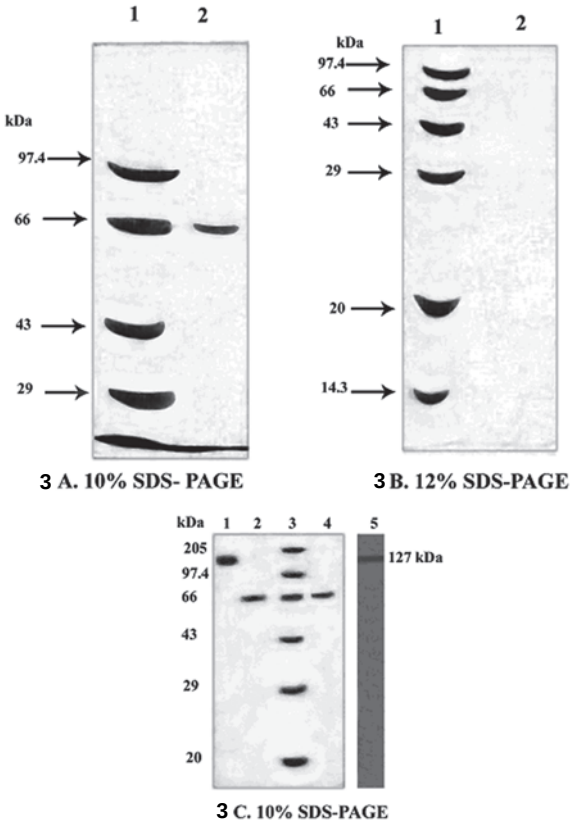

Fig 3. Molecular mass of purified ginger rhizome PPO. A & B: Lane 1, protein molecular weight marker; lane 2, purified ginger rhizome PPO; C. Four aliquots of purified PPO were loaded. Lane 1, PPO in sample buffer devoid of reducing agent and without heating; lane 2, PPO heated in presence of reducing agent; lane 3, protein molecular weight marke; lane 4, PPO heated in absence of reducing agent; lane 5, PPO without reducing agent and without heating were loaded and electophoresed. (The first four lanes were stained with Coomassie brilliant blue R-250. The last lane from the same gel was stained separately for PPO activity).

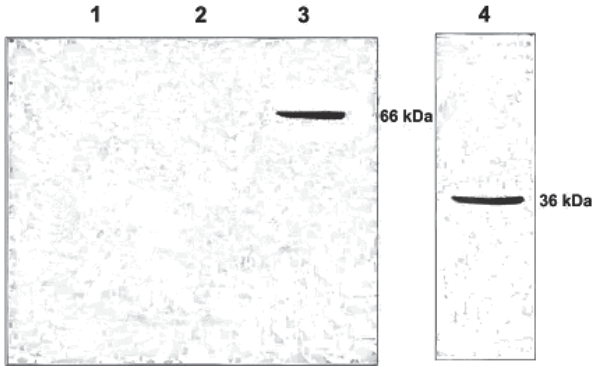

Fig 4. Western blot analysis of ginger PPO. Lane 1 and 2, ginger leaf crude extracts from 2 months and 4 months old plants; lane 3, purified ginger rhizome PPO; lane 4, potato tuber crude extract.

accessions (Fig. 1A) and cross reactivity study by ELISA techniques were in agreement with this finding. The purified PPO may have an antigenic determinant different from that of leaf PPOs that limits antibody cross reactivity. In the present experiment, in lane loaded with potato tuber extract, the ginger PPO antibody detected a lower molecular mass protein of approximately 36 kDa. In potato, a PPO of sub unit mass 36 kDa was reported by Balsingam & Ferdinand (1970).

Cross reactivity with other crops as evaluated by ELISA

Ginger rhizome PPO antiserum showed a high degree of cross reactivity with potato, radish and yam tuber extracts in ELISA. Significant cross reactivity was observed with brinjal, apple and banana fruit extracts also but no cross reactivity was observed with sweet potato and also with mango ginger which comes under the same family.

Effect of temperature on stability and o-Diphenolase activity

The purified PPO showed maximum o-diphenolase activity at 60°C and it was found to be thermo-tolerant. Incubation at 45°C and 60°C for 45 min activated the enzyme significantly and it appeared to be tolerant of even higher temperature. Residual PPO activity after incubation at 75°C for 45 min was 89% of that at 60°C. Incubation at 90°C rapidly inactivated the PPO and after 45 min, only 34% of the activity at 60°C was retained indicating that the purified PPO is heat labile at this temperature. Many plant PPOs were shown to be very tolerant to heat treatments such as a latex PPO from *Hevea brasiliensis* Willd. (Wititsuwannakul *et al*. 2002) and PPO-1 from hybrid poplar leaves (Wang & Constable 2003).

Effect of pH on stability and o-Diphenolase activity

The purified PPO isoform showed maximum activity at pH 4.5 (activity 174% of that at pH 6.5) and the enzyme activity sharply declined above pH 7.5. Storage for 24 h at 4°C at acidic pH (3.5 to 5.0) and also at alkaline pH (9 to 9.5) activated the enzyme whereas storage above pH 10.5 resulted in significant loss of activity. The presence of a broad optimum in the pH curve of PPO activity was reported in some plant PPOs (Wititsuwannakul *et al*. 2002; Wang & Constable 2003).

Effects of chlorides

Lower concentrations of $MgCl_2$ (1mM) and $CaCl_2$ (0.5mM) were found to activate the enzyme whereas higher concentrations (10 mM) reduced the activity (Table 2). Halides such as $CaCl_2$, $MgCl_2$ and NaCl were inhibitory to *Colocasia antiquorum* PPO at specific concentrations (Yemenicioglu *et al*. 1999).

Table 2. Effect of chlorides on ginger PPO activity

Chemical	Concentration (mM)	PPO activity (U mg^{-1} protein)[a]
$CaCl_2$	0.0	14.3
	0.5	16.7
	1.0	16.1
	5.0	11.7
$MgCl_2$	0.0	15.2
	0.5	15.4
	1.0	18.4
	5.0	12.5
NaCl	0.0	15.2
	0.5	14.2
	1.0	11.7
	5.0	9.2

[a]One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmol of 4-methyl-o-benzo-quinone min^{-1}.

Effect of thiols and reducing agents

L-Cysteine HCl, ascorbic acid and sodium metabisulfite showed very high inhibition of ginger rhizome PPO at 0.5 mM concentration. At 1 mM concentration in the reaction mixture, L cysteine HCl showed a lag phase of 70 min whereas L-ascorbic acid showed a lag phase of 56 min when 4-methyl catechol was used at 20 mM concentration in the reaction mixture (Table 3).

Table 3. Effect of inhibitors on PPO activity

Inhibitor (µM)	[a]Activity (U)	Lag phase (min)
Ascorbic acid		
Control	16.9	-
25	17.0	-
50	16.0	-
100	9.8[b]	0.4
250	8.0[b]	5.0
500	3.6[b]	16.0
1000	2.2[b]	56.0
Cysteine Control	17.0	-
25	16.4	-
50	11.1	-
100	4.9 [b]	0.67
250	4.7 [b]	11.0
500	3.6 [b]	25.0
1000	2.8 [b]	70.0

[a]One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmol of 4-methyl-o-benzoquinone min^{-1}; [b]Activity recorded after the lag phase using 20 mM 4- methyl catechol as substrate.

Addition of inhibitors to the raw ginger can stop discoloration at different stages of processing such as peeling, cutting and pulping. But the inhibition will be lost once the inhibitor is totally used up and hence after processing, vacuum packaging or appropriate storage conditions are required to avoid discoloration. Inhibitory properties of these compounds have been reported in other PPOs (Yemenicioglu *et al*. 1999; Dogan *et al*. 2005). L-Cysteine HCl, ascorbic acid and chlorides are of potential use in ginger processing since most other PPO inhibitors are toxic to humans.

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