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ACTIVATION OF PURIFIED POLYPHENOL OXIDASE AND LACCASE OF CHICKPEA CV ICCV10 BY SODIUM DODECYL SULFATE

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ABSTRACT
The effect of sodium dodecyl sulfate (SDS) was studied on the purified polyphenol oxidases (PPO-1) and PPO-II/ laccase of chickpea cv ICCV 10 infected by *Fusarium oxysporum* f. sp. *ciceri*. Catachol and ABTS were found to be the the best substrates for PPOs and laccase enzymes respectively.PPO-I and PPO-II/laccase activities were increased by 10-40% at 10 μM of SDS. Benzoic acid was shown to be a competitive inhibitor in absence of SDS, where as in presence of SDS the type of inhibition was found to be noncompetitive. Therefore SDS is an activator of PPOs and lacaase enzymes that can probably change latent form of enzyme to active form, so increases the activities of PPOs.

INTRODUCTION

Polyphenol oxidase (PPO) (E.C. 1.14.18.1) also known as phenolase, catechol oxidase, catecholase, monophenol oxidase, o-diphenol oxidase and orthophenolase based on their substrate specificity, is widely distributed in plants and fungi (Mayer, 2006; Kaur *et al.*, 2010; Asha *et al.,* 2011; Pawar and Ingle, 2014). PPOs have been purified and characterized from a wide range of plant species and a variety of tissues, and activity levels using common substrates vary widely (Constabel and Barbehenn, 2008; Gayatridevi *et al.*, 2014). Based on its association with browning reactions in crop plants, PPO has been characterized in a wide variety of food plants including banana, wheat, quince, and avocado, and a number of chemical inhibitors have been identified (Yoruk and Marshall, 2003; Mayer, 2006). In parallel, the potential roles for PPO in plant defense against pests have motivated many studies on PPO in an ecological context, though few of these have used a transgenic approach.

Earlier we (Gayatridevi *et al.,* 2014) have purified and characterized native and induced polyphenol oxidases from the chickpea cv ICCV 10 infected by *Fusarium oxysporum* f. sp. *ciceri*.Futher we have shown that the induced PPO was associated with the laccase activity and their activities were increased in presence of salicylic acid. Taken together, we concluded that the constitutive and induced PPOs with distinct kinetic properties and differential sensitivity to SA, suggested that each isoforms plays a different physiological role in providing immunity to the chickpea plant. Native PPO-I provide natural defense by oxidation of endogenous phenolic pool to quinones, while a novel bifunctional PPO-II/laccase provide additional induced defense by synthesizing quinones as well as lignin during the Foc infection. The combinations of both enzymes provide enhanced resistance against Foc in chickpea plant.

In plants, Polyphenol oxidases have been known to exist in multiple forms and found to be inactive or latent state in the thylakoid membranes. PPOs can activated by different treatments such as acid and base shock, urea and SDS. SDS was shown to be as an activator at very low levels for different enzymes like pancreatic lipase, pyruvate oxidase and *Xenopus tyrosinase* (Moore and Flurkey, 1990) in contrast with the many that are inactivated by it. It was known that the activation of PPO from crude broad bean leaf by SDS occurred at below 1 mM SDS (Kenten, 1958). Extending these observations further, Robb *et al.* (1964) observed that this activation process is reversible and that prolonged incubation in the presence of SDS leads to a loss of activity. Laveda *et al.* (2001) demonstrated the total reversibility of the SDS activation of latent peach PPO by SDS entrapment with cyclodextrins. Hutchenon *et al.* (1980) have reported the activation of the broad bean PPO by SDS, pH and fatty acids. Similar observations were also found in spinach PPOs. On the other hand, there have been several reports on the extraction, purification and kinetics of PPOs from different sources in presence of SDS (Jukanti *et al.,* 2003; Saeidian *et al.,* 2007). In

the present investigation, we report the effect of sodium dodecyl sulfate (SDS) on the activities of PPO and laccase of chickpea cv ICCV 10.

MATERIALS AND METHODS

Plant materials and treatments

Chickpea (*Cicer arietinum* L.) a resistant genotype ICCV 10 to wilt caused by *Fusarium oxysporum* f. sp. *ciceri* were procured from the Agriculture Research Station, Gulbarga, India. SA or Foc treatments of seeds and germination experiments were performed as described in our earlier report (Gayatridevi *et al.,* 2014). All other reagents used were of analytical grade.

Chickpea enzyme extraction

Weighed sample (50 g) (chickpea shoots) was ground in a pre-chilled pestle and mortar in 0.1 M phosphate buffer, pH 7.0 containing 1% Triton X-100, PMSF (1mM) and 1% PVP and centrifuged at 12,000 g for 25 min at 4° C. The supernatant obtained was used as a source of enzyme for analysis of PPO activity. The protein concentration in the supernatant was determined according to Lowry *et al*. (1951) method using bovine serum albumin as a standard.

Polyphenoloxidase assay

Enzyme activity (PPO) was determined by measuring the increase in absorbance at 420 nm using catechol as a substrate. One unit of PPO activity was expressed as the amount of enzyme that causes an increase in absorbance of 0.001 ml^{-1} min^{-1}(Mayer *et al.,* 1965)

Laccase assay

Laccase activity was determined with ABTS [2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonate)] as the substrate (Bourbonnais and Paice, 1990). The reaction mixture (1 ml) containing 0.1 ml enzyme and 0.1 ml ABTS (1mM) and dissolved in 50 mM phosphate buffer (pH 7.0), oxidation of ABTS was monitored by following the increase in absorbance at 436 nm ($\varepsilon 29 \pm 3$ mM^{-1} cm^{-1}). One unit of laccase activity was defined as the amount of enzyme that oxidized 1 μmol of substrate/min.

Purification of chickpea PPOs

A detailed procedure for the purification and characterization of PPO-I and PPO-II/laccase from the chickpea cv ICCV10 treated with Foc or salicylic acid was described in our earlier report (Gayatridevi *etal.,* 2014).

Substrate specificity

Purified PPOs activities were assayed using monohy droxyphenols (p-cresol and DL-tyrosine), dihydroxyphenols (catechol and 4-methy lcatechol), tri-hydroxy phenols (pyrogallol) in buffers at optimum pH values. The rate of the reaction was measured in terms of the increase in absorbance at the wavelength of maximum absorption for the corresponding product. The assays were carried out in cuvettes (3 mL) containing the buffer solution at pH optimum.

Effect of SDS on PPO

The SDS solutions (0 to 100 μM) were prepared in 0.1M sodium phosphate buffer (pH 7.0). The enzyme assay solution contained different concentrations of the buffered SDS solutions and substrates as explained above.

Determination of type of inhibition

Benzoic acid was examined for their effectiveness as inhibitors of PPO-I and PPO-II using catechol as substrate in presence of SDS (10 μM). In each case, the type of inhibition was deduced from Lineweaver-Burk double reciprocal plots.

Statistical analysis

All optimization experiments were carried out in duplicate to check the reproducibility of results. The results presented here are the average values of duplicate determinations $\pm$ SD.

RESULTS

Substrate specificityfor PPOs and laccase

Monohydroxyphenols (cresol and tyrosine) were not oxidized by the chickpea PPOs. *O*-Diphenols (catechol) and triphenols (pyrogallol) were oxidized significantly. This result is consistent with the previous reports indicating that catechol is usually the best substrate for PPOs. Dopamine, L-Dopa and gallic acid were not oxidized by chickpea PPOs (Table 1), whereas for laccase, ABTS and guiacol were the best substrates; however laccase was not oxidized catechol and tyrosine as they are not good substrates (Table 2).

Effect of SDS and benzoic acid on the purified PPO-I, PPO-II and laccase

The effect of SDS on both the purified PPO-I and PPO-II was studied. PPO-I, PPO-II and laccase activities were increased by 10 to 40% at 10 μM of SDS (Table 3). Benzoic acid at 1 mM concentration inhibited PPO-I competitively (Fig. 1), whereas in presence of SDS (10 μM) the inhibition was found to be noncompetitive. Similarly, PPO-II and laccase were also inhibited by benzoic acid (Table 4).

DISCUSSION

The role of PPOs in plant defense against herbi-vores and pathogens has been reported (Jukanti 2003; Thipyapong*et al.,* 2004; Thipyapong *et al.,* 2007). The possible mechanism of the role of PPOs in the plant defense system is unknown,

Table 1: Substrate specificity of chickpea PPO

Substrate	[S](mM)	PPO-I	PPO-II
		Activity relative to catechol (%)	
Catechol	1	100	100
L-Tyrosine	1	0	0
pyrogallol	1	62	50
Dopamine	1	0	0
L-Dopa	1	0	0
Gallic acid	1	0	0
P-Cresol	1	0	0

Data are mean values from triplicate assays.

Table 2: Substrate specificity of purified laccase from chickpea

Substrates	Concentration (mM)	Relative activity (%)
p-Phenylenediamine	1	1.00
Guaiacol	10	l0.95
Catechol	1	0.00
Tyrosine	1	0.00
ABTS	1	100.00

Data are mean values from triplicate assays

Table 3: Effect of different concentrations of SDS on the purified PPO-I and PPO-II and Laccase activity

SDS (μM)	Relative activities of (%)*		
	PPO-I	PPO-II	Laccase
0	100	100	100
5	110	118	121
10	128	138	141
15	125	133	138
20	121	128	129
25	116	121	120

*Enzyme activities were determined with their respective substrates as described in materials and methods

Table 4: Type of inhibition of PPO-I, PPO-II and Laccase by benzoic acid in presence of SDS (10 μM)

Enzyme*	Type of inhibition	
	Absence of SDS	Presence of SDS
PPO-I	Competitive	Noncompetitive
PPO-II	Competitive	Noncompetitive
Laccase	Competitive	Noncompetitive

*enzyme activities were determined with their respective substrates as described in Materials and methods.

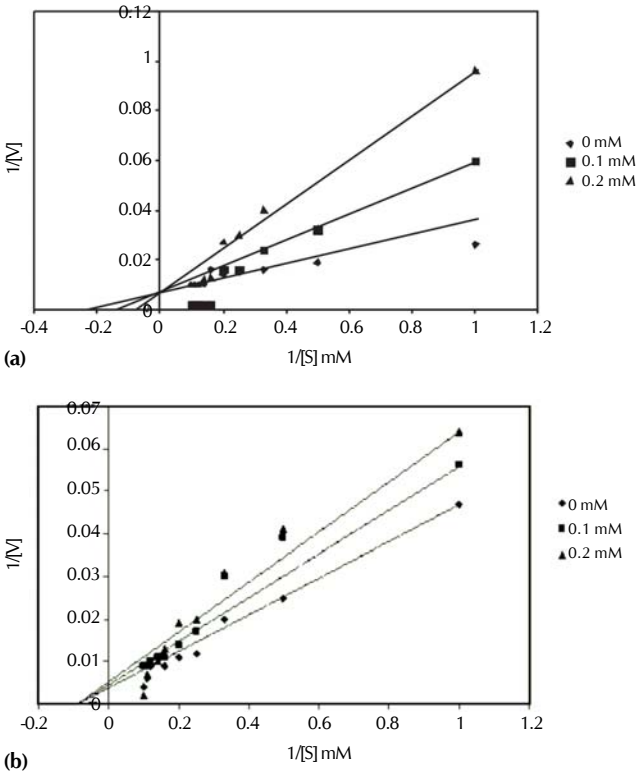

Figure 1: Inhibition of PPO-I by Benzoic acid at pH 6.8 and 30ºC with respect to catechol (a) in absence of SDS; b). in presence of SDS (10 μM)

and consequently remains hypothetical, but there have been some attempts to provide evidence. Moreover, the increase in PPO activity in host-pathogen interaction and the binding of o-quinones to proteins can induce an antinutritive defense (Jukanti, 2004). PPOs are also known to oxidize phenols and produce fungi-toxic quinones, and in this way provide a plant defense against pathogenic fungi (Lattanzio, 2003;Lei *et al.,* 2004). Catecholases, laccases and cresolases in plants can exist with low or undetectable activity and should therefore be enhanced by fatty acids, mild heat, acid or alkali, proteolytic enzymes such as trypsin, detergents such as sodium dodecyl sulfate (SDS) or extended incubation (Schmitz *et al.,* 2008).The use of SDS as an activating agent in the case of broad bean PPO (Moore and Flurkey, 1990) and red and green tomotos (Saeidian and Rashidzadeh, 2013) has been reported.

In the present study, it was found that the PPOs are activated to10-40% by SDS at 10 μM. The inhibition pattern was also changed from competitive to non competitive by benzoic acid in presence of SDS. It has been reported that SDS binding was related to enzyme stabilization and binding to the enzyme with high affinity. Isotherms for SDS binding were correlated with SDS-activation/stabilization and suggested that activation was a result of SDS binding to the enzyme. The concentration of SDS needed for maximum activation was related to the amount of SDS monomers in solution. The sigmoidal nature of SDS activation from all the reported (Moore and Flurkey, 1990) was suggested of a cooperative interaction between SDS binding and activation and perhaps indicates a conformational change associated with binding of the SDS monomer.

The reason for increase of activity of PPO is that probably polyphenol oxidase exists in latent form, therefore in presence of SDS, binds to the protein chain with its hydrocarbon tail, exposing normally buried regions of latent form of polyphenol oxidase and changes its conformation to active forms in low concentrations of SDS. But with more increase in concentration of SDS, It would be a denaturant for PPO, So the detergent wraps around the polypeptide backbone and coating the protein chain with surfactant molecules. Therefore in high concentration of SDS, PPO activity gradually decreased and in more concentration, PPO activity probably will reach to zero because of denaturing of enzyme.

In conclusion, the protective role of PPOs can be increased by low concentrations of SDS, against herbivores. PPOs play a crucial role in oxidizing phenolic compounds and converting them into reactive defensive molecules. The molecular mechanisms of this strategy are largely unknown, although it has been suggested that PPOs affect brown coloring and that this in itself plays a role in defending the plant against various biotic and abiotic stresses (Pourcel *et al.,* 2007).

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