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Polyphenol oxidases from latex of *Hevea brasiliensis*: purification and characterization

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Abstract

Polyphenol oxidase (PPO) was isolated from the B-serum obtained after repetitive freeze-thawing of the bottom fraction isolated from ultracentrifuged fresh latex. The B-serum was subjected to acetone precipitation and CM-Sepharose chromatography, affording two PPOs, PPO-I and PPO-II, which, upon SDS-PAGE, were 32 and 34 kDa, respectively. Both PPOs possessed the same pI (9.2), optimum pH (7) and optimum temperature (35–45 °C). They are stable up to 60 °C and active at broad pH ranges from 4–9. The K_m values of PPO-I for dopamine, L-dopa and catechol as substrates are 2.08, 8.33 and 9.09 mM, while those for PPO-II are 2.12, 4.76 and 7.14 mM, respectively. Among various PPO inhibitors tested, 4-hexylresorcinol was the most potent. Anionic detergents were among the most effective activators of the enzymes, while cationic and nonionic detergents showed little and no effect on the PPO activities, respectively.

Keywords: *Hevea brasiliensis*; Euphorbiaceae; Rubber latex; Polyphenol oxidase

1. Introduction

Polyphenol oxidase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1), also known and reported under various names (tyrosinase, phenolase, catechol oxidase, catecholase, monophenol oxidase, *o*-diphenol oxidase and orthophenolase) based on substrate specificity (Mayer and Harel, 1979; Vaughn and Duke, 1984; Mayer, 1987), is widely distributed in plants and fungi (Mayer, 1987; Mayer and Harel, 1979; Vaughn and Duke, 1984; Vaughn et al., 1988; Sherman et al., 1991). In higher plants the enzyme has been localized to the thylakoid membranes of chloroplasts and other plastid organelles. Polyphenol oxidase (PPO) is frequently reported as a latent enzyme, which can be activated in vitro by a number of different factors and treatments such as detergents (Swain et al., 1966; Kenten, 1958; Mayer and Friend, 1960; Moore and Flurkey, 1990), proteases (Tolbert, 1973; King and Flurkey, 1987), low and high pH levels (Kenten, 1957) and exposure to fatty acids in the incubation mixtures (Golbeck and Cammarata, 1981). In this paper, the purification and characterization of PPO from the natural rubber latex of *Hevea brasiliensis* is reported.

2. Results and discussion

2.1. Distribution and possible function of the latex PPO

Polyphenol oxidase (PPO) was earlier reported to be present in both *Hevea* latex lutoid and Frey-Wyssling particles (Coupé et al., 1972). We found that the latex PPO activity in lutoids was 5- to 34-fold higher than that of the Frey-Wyssling particles (Table 1). Since the latex bottom (lutoid) fraction was reported to contain several pathogenesis-related (PR) proteins, such as chitinases,

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β-1,3-glucanase and hevein (Churngchow et al., 1995; Subroto et al., 1996; Van Parijs et al., 1991), the presence of PPO in the same fraction may be pathogenesis-related as well. The transcripts of several lacticifer specific genes, highly expressed in *Hevea* lacticifers than in the leaves, were previously shown to include these PR proteins (Kush et al., 1990; Broekaert et al., 1990; Chye and Cheung, 1995). The accumulation of PR proteins in the lutoids is assumed to be a wound response from tapping of rubber trees for harvesting latex. The wound-induced activation of specific genes was also shown in *Hevea* system to be due to tapping wound (Kush, 1994). Hence, the PPO accumulating in lutoids, as shown in this study, may also serve as one of the wound-induced enzymes participating in defense against pests and pathogens. This is similar to what was reported in tomato (Thipyapong and Steffens, 1997; Thipyapong et al., 1995), and is consistent with the proposed defensive role of PPO in plants (Mayer and Harel, 1979; Mayer, 1987). Accordingly, the highest level of latex PPO was found in latex of GT 1 clone (Table 1) which is the most disease resistant *Hevea* clone grown in Thailand. Moreover, the brown color development on the cut or sliced fruits and vegetables, is also similarly observed on dry rubber sheets prepared from milky white fresh latex. The white color of wet rubber sheet is gradually changed into brown color during the open-air drying period. This is presumably due to the latex PPO activity catalyzing oxygen-dependent oxidation of phenols to reactive quinones, resulting in the brown color formation in the dry rubber sheets. Generally, the brown color of dry rubber sheet derived from GT 1 latex is relatively darker than that obtained from the RRIM 600.

2.2. Purification of the latex PPO

The PPO in crude lutoid extract (B-serum), released from the bottom fraction by freezing and thawing, was subjected to acetone precipitation and ion-exchange chromatography (Table 2). A 6-fold increase in PPO activity was obtained after acetone treatment. Similar activation effects were previously observed with whole chloroplasts of sugar beet (Mayer and Friend, 1960). The increase in PPO activity may result from limited proteolysis, by proteases present in the B-serum (Pujarniscle, 1968), during the PPO isolation. The activity of PPO in B-serum dialyzed against buffer containing protease inhibitors was lower than that containing no protease inhibitors, and much lower than the B-serum sample kept under the same condition at 4 °C for 24 h, respectively (Table 3), which further supports PPO activation by proteolysis. After CM-Sepharose column chromatography, a major PPO-I peak (ca 70% of total PPO activity) and a minor PPO-II peak, were obtained from stepwise elution with buffer containing 0.1 and 0.2 M NaCl, respectively (Fig. 1). The specific activity of the eluted PPO-II was about 2.5-fold higher than that of the PPO-I (Table 2).

2.3. Molecular weight of the latex PPO

Under SDS-PAGE, PPO-I and PPO-II were revealed as partially purified and purified ca 32 and 34 kDa

Table 1
Distribution of PPO activity in the ultracentrifuged fresh latex

Rubber clone	PPO activity (nkat/ml latex)[a]	
	Lutoid	Frey-Wyssling
RRIM 600	7.33	0.21
GT 1	9.21	0.65
KRS 21	4.34	0.77

[a] Minimal PPO activity was detected in C-serum and rubber fraction.

Table 2
Purification protocol of the latex PPO

Step	Total protein (g)	Total activity (nkat)	Specific activity (nkat/mg)	Yield (%)
B-serum	2.71	7138	2.63	100
Acetone precipitation	0.63	42,920	68.13	601
CM-Sepharose				
PPO-I	0.10	23,399	233.99	328
PPO-II	0.02	10,182	509.10	143

Table 3
Limited proteolysis effect on B-serum PPO activity

Treatment	Relative PPO activity (%)
1. 4 °C for 24 h	100
2. Dialysed against 50 mM Tris–HCl, pH 7 at 4 °C for 24 h	69
3. Same as for 2 but buffer contained 1 mM EDTA and 1 mM PMSF	42

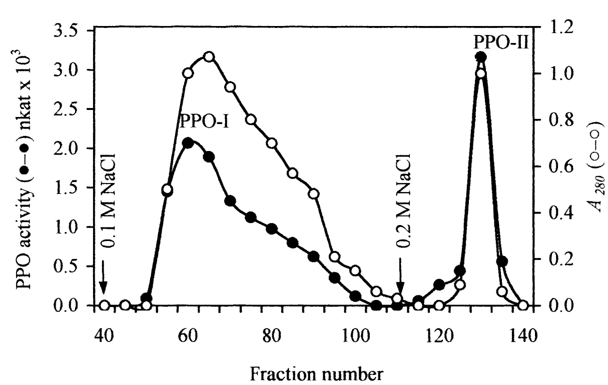

Fig. 1. Chromatographic elution profile of PPO on CM-Sepharose column. The latex PPO solution was prepared from the dissolved pellet fraction obtained after B-serum acetone precipitation and used for column chromatography, as described in Section 3.

enzymes, respectively (Fig. 2). Gel filtration chromatography showed both enzymes to be monomeric proteins with M_r of 32 and 34 kDa. These M_r values of latex PPO are slightly lower than that reported for the carrot cell culture (Soderhall and Soderhall, 1989).

2.4. pI of the latex PPO

Both PPOs possessed the same pI of about 9.2 (Fig. 3). They therefore belong to the major class of positively charged B-serum basic proteins (Southorn and Yip, 1968), but are different from the majority of acidic PPOs, reported in other plant tissues as well as fungi (Soderhall and Soderhall, 1989; Janovitz-Klapp et al., 1989; Shin et al., 1997; Motoda, 1999).

2.5. Effect of pH and temperature on the latex PPO activity

The same optimum pH of 7 and optimum temperature of 35–45 °C were found for both enzymes, respectively (Figs. 4A and 5A). The latex PPOs' optimum pH value is higher than those found among the acidic PPOs (Shin et al., 1997; Motoda, 1999; Kader et al., 1997). They were stable in a broad pH range from 4 to 10, with more than 80% of the original activities retained at the extreme pH of 4 and 10 (Fig. 4B), which is similar to that reported in the sunflower seeds (Raymond et al., 1993). Both PPOs showed thermal stability up to 60 °C with almost 80% activity remaining after heat pretreatments prior to the enzyme assays (Fig. 5B).

2.6. Substrate specificity and enzyme kinetics

Different K_m values of PPO-I and PPO-II, using dopamine, L-dopa and catechol as substrates (Table 4), were obtained from the Lineweaver-Burk plots. The K_m values of PPO-I for dopamine, L-dopa and catechol are 2.08, 8.33 and 9.09 mM, while those for PPO-II are

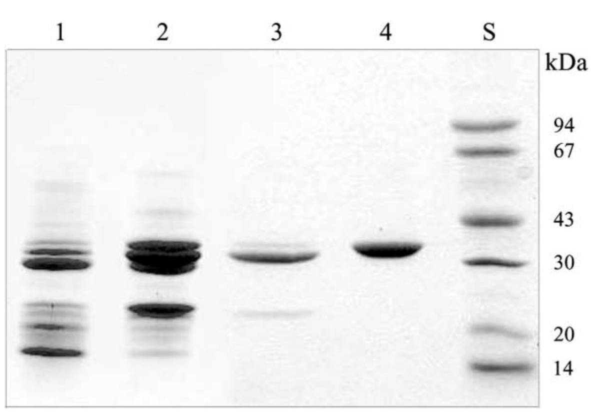

Fig. 2. SDS-PAGE of the purified latex PPO. Lane 1: B-serum (100 µg); lane 2: 30–50% acetone pellet fraction (100 µg); lane 3: PPO-I (20 µg); lane 4: PPO-II (20 µg); lane S: standard protein markers.

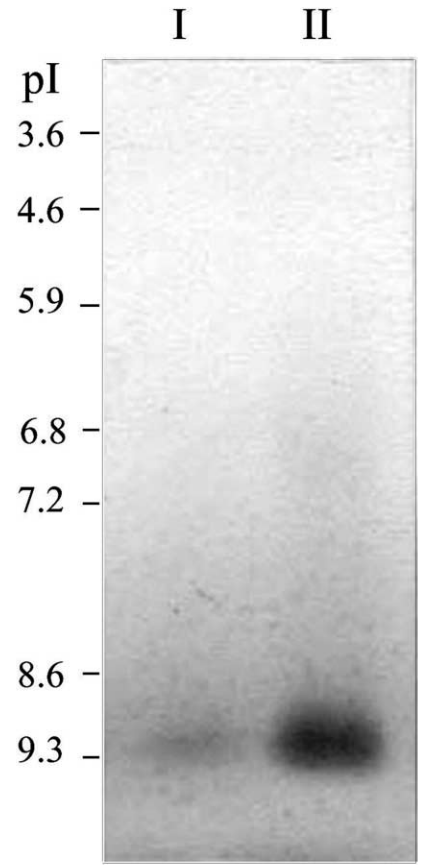

Fig. 3. Isoelectric focusing gel electrophoresis of PPO. Lane I: PPO-I (4 µg); lane II: PPO-II (4 µg).

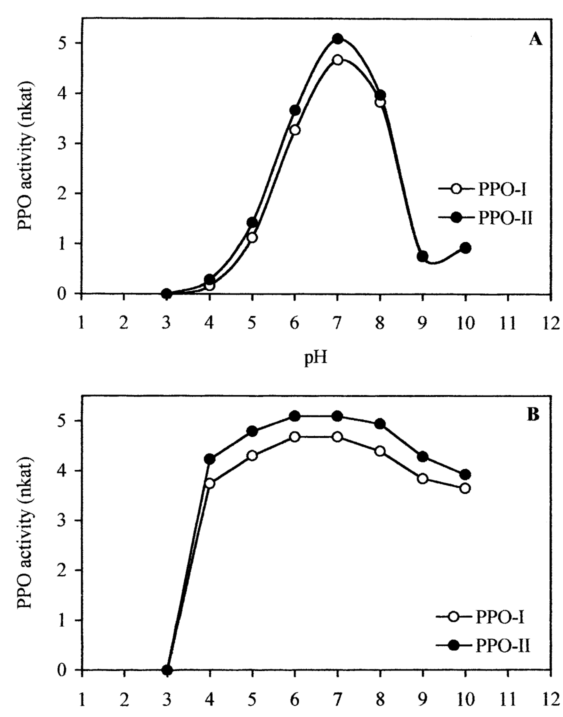

Fig. 4. pH optimum (A) and pH stability (B) of PPO-I and PPO-II fractions from CM-Sepharose column.

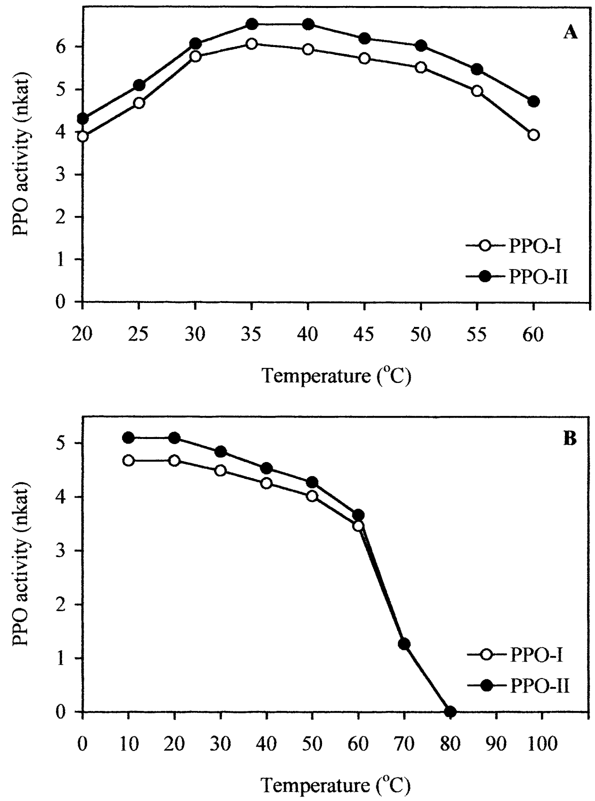

Fig. 5. Optimum temperature (A) and thermal stability (B) of PPO-I and PPO-II fractions from CM-Sepharose column.

Table 4
Kinetic studies on the K_m of PPO-I and PPO-II

Substrate	PPO-I	PPO-II
Dopamine (mM)	2.08	2.12
L-Dopa (mM)	8.33	4.76
Catechol (mM)	9.09	7.14

2.12, 4.76 and 7.14 mM, respectively (Table 4). Both enzymes have lower K_m values of about 4-fold toward dopamine as compared to catechol substrate. Among several *ortho*-dihydroxyphenol substrates tested, PPO-II revealed its highest relative substrate specificity toward L-dopa (3.31 nkat) and lowest toward catechin of 0.74 nkat (Table 5), similar to those reported in spinach chloroplast (Sheptovitsky and Brudvig, 1996). Moreover, the latex PPO-II exhibited no substrate specificity towards either monophenols or 3′,3′-diaminobenzidine, a suitable substrate for peroxidase and laccase. Hence, it is more likely to be a diphenol oxidase (EC 1.10.3.1) than a monophenol oxidase.

2.7. *Effect of inhibitors and activators*

The effect of various inhibitors on latex PPO, obtained after acetone fractionation, with dopamine as substrate was determined (Table 6). Several compounds reported as PPO inhibitors (Raymond et al., 1993; Anosike and Ayaebene, 1981; Halim and Montgomery, 1978) were also shown to have inhibitory effect on the latex PPO. 4-Hexylresorcinol, a specific inhibitor for PPO in antibrowning, was the most potent inhibitor and able to inhibit activity up to 84% at low concentration (0.1 mM), although the results from inhibitor studies (Raymond et al., 1993; Anosike and Ayaebene, 1981; Halim and Montgomery, 1978; Golan-Goldhirsh and Whitaker, 1984) in other plant tissues (tuber, seed, fruit and mushroom) showed the thiol reagents as the most effective inhibitors for those enzymes. The level of latex PPO activity inhibited by 4-hexylresorcinol was, however, found to be higher than those with the thiol reagents either under equal or higher concentrations. NaN_3, a broad spectrum peroxidase inhibitor, at 10 mM was unable to inhibit the latex PPO. NaCl, a browning inhibitor and observed to be the weakest PPO inhibitor in several plant tissues (Kavrayan and Aydemir, 2001; Yang et al., 2000; Raymond et al., 1993; Anosike and Ayaebene, 1981; Halim and Montgomery, 1978), was also unable to inhibit latex PPO (Table 6). The results on enzyme specificities towards specific inhibitors as well as substrates (Table 5) suggested that it is a PPO, not peroxidase or laccase.

Table 5
Substrate specificity of the latex PPO-II

Substrate	[S] (mM)	Activity (nkat)	Activity relative to dopamine (%)
Dopamine	5	2.53	100
L-Dopa	5	3.31	131
Catechol	5	1.56	62
Catechin	5	0.74	29
Benzoic acid	5	0	0
p-Coumaric acid	5	0	0
Tyrosine	5	0	0
3′,3′-Diaminobenzidine	5	0	0

Table 6
Comparative study on effects of various inhibitors on latex PPO activity[a]

Inhibitor	mM	Inhibition (%)
None	0	0
4-Hexylresorcinol	0.1	84.10
Dithiothreitol	0.1	66.76
Sodium metabisulphite	0.1	20.89
2-Mercaptoethanol	0.1	17.60
Ascorbic acid	0.1	3.71
Thiourea	1.0	33.41
Resorcinol	1.0	13.21
NaN_3	10	0
NaCl	10	0

[a] The enzyme (20 μg) was preincubated with various inhibitors as indicated at room temperature for 5 min before starting PPO assay by addition of dopamine (10 mM final concentration).

The modulation of latex PPO activity by detergents was studied using anionic (SDS and DOC), cationic (CTAB) and nonionic (Triton X-100 and Tween 20) detergents (Table 7). Anionic detergents were found to be more effective in the activation of latex PPOs than the cationic one. The presence of SDS (10 mM) resulted in a maximum of 12-fold increase in the catalytic efficiency of the activated enzyme, comparable to that described for banana and broad bean PPOs (Moore and Flurkey, 1990; Sojo et al., 1998), but higher than those described for table beet and spinach PPO (Escribano et al, 1997; Sánchez-Ferrer et al., 1989). Similarly, DOC (10 mM) was also able to activate the enzyme by 5.2-fold, which is higher than that described for sugar beet (Mayer and Friend, 1960). The level of latex enzyme activation in the presence of cationic CTAB (10 mM) was insignificant (1.6-fold), which is similar to that described for banana PPO (Sojo et al., 1998), but different from spinach PPO where CTAB acted as the enzyme inhibitor (Sánchez-Ferrer et al., 1989). In contrast to ionic detergents, the latex PPOs could not be activated by nonionic detergents, similar to those found with the latent broad bean and sugar beet PPOs (Kenten, 1958; Mayer and Friend, 1960). The ability of SDS to activate the enzyme may involve alterations of its enzymatic and physical characteristics, as well as a limited conformational change due to binding of small amounts of SDS. This may induce or initiate the activation of the latent enzyme as earlier suggested (Moore and Flurkey, 1990).

3. Experimental

3.1. Chemicals

CM Sepharose CL-6B, dopamine, L-dopa, catechol, catechin, dithiothreitol, sodium azide, 2-mercaptoethanol, 4-hexylresorcinol, resorcinol, 3′,3′-diaminobenzidine, sodium dodecyl sulfate (SDS), deoxycholate (DOC) and hexadecyl trimethyl-ammonium bromide (CTAB) were obtained from Sigma. Ascorbic acid, sodium metabisulphite, thiourea, sodium chloride, Triton X-100 and Tween 20 were purchased from Merck. All other chemicals were of analytical grade.

Table 7
Comparative study on effects of different detergents on latex PPO activity[a]

Detergent	nkat		% of control	
	1 mM	10 mM	1 mM	10 mM
None	8.64	8.64	100	100
SDS	69.11	103.42	800	1200
DOC	8.52	44.85	100	520
CTAB	13.73	12.78	160	150
Triton X-100 (1%)	7.93	90		
Tween 20 (1%)	7.93	90		

[a] Dialysed B-serum (0.2 mg) was used for the assay.

3.2. Plant material (collection and fractionation of latex)

Freshly tapped latex was collected in an ice-chilled beaker from regularly tapped trees of RRIM 600 clone or as specified. The latex was fractionated by ultracentrifugation (59,000 *g*, 45 min, 4 °C) to give four distinct layers. The top layer was a white creamy thick layer of rubber, the next thin layer underneath was yellowish and called Frey-Wyssling, the middle layer was a clear solution called C-serum, and the pellet was the bottom (lutoid) fraction. The Frey-Wyssling particles and the bottom fraction from three rubber clones (RRIM 600, GT 1 and KRS 21) were separated and used to study distribution of PPO activity.

3.3. PPO activity assay

PPO activity was spectrophotometrically monitored by following the oxidation of 5 mM substrate (dopamine, 470 nm; L-dopa, 475 nm; catechol, 410 nm; catechin, 380 nm; benzoic acid, 410 nm; *p*-coumaric acid, 400 nm; tyrosine, 472 nm or 3′,3′-diaminobenzidine, 410 nm) in 50 mM phosphate buffer, pH 7, at 25 °C. The total assay volume was 1 ml. The linear portion of the absorbance vs time curve was used to determine the initial rates. PPO activity was expressed in nkat (nmol substrate converted/s).

3.4. Purification of latex PPO

B-serum was prepared from isolated bottom fraction (80 g) by repetitive (4–5 times) freeze-thawing at −20 and 37 °C. The supernatant, which is the B-serum, was obtained after centrifugation at 10,000 *g* for 20 min (4 °C) and brought to 30% saturation with acetone, under continuous stirring at 4 °C for 10 min. The mixture was centrifuged and the pellet discarded. Acetone was further added to the resulting supernatant to reach 50% saturation and again stirred at 4 °C for 10 min. The acetone pellet was collected by centrifugation, freed of residual acetone (by blowing under nitrogen gas), dissolved in a minimum volume of 50 mM Tris–HCl buffer, pH 7 and used for further purification.

A CM-Sepharose CL-6B column (2.5 × 24 cm) was pre-equilibrated with 50 mM Tris–HCl pH 7 at a flow rate of 18 ml/h at 4 °C. After loading the resuspended acetone precipitate solution, the column was washed with the same buffer until the absorbance at 280 nm was below 0.005. The column was then subjected to stepwise-elution by using the same buffer containing 0.1 and

0.2 M NaCl, respectively. The fractions containing high PPO activity were pooled, desalted and concentrated for further characterization.

3.5. Gel filtration for molecular weight estimation

Gel filtration for measuring the molecular weight of native PPO was carried out on a Sephadex G-100 column (1×90 cm). A 1-ml sample of the CM-Sepharose-purified PPO was loaded on Sephadex G-100 column equilibrated with 50 mM Tris–HCl, pH 7. Flow rate was 9 ml/h and 1-ml fractions were collected and their A at 280 nm and PPO activity measured. The standard markers used were albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). PPO-I and PPO-II molecular weights were determined from the curve plotted between relative log molecular weight of the standard markers against their K_{av} values.

3.6. Effect of pH on PPO activity

Enzyme activity, as a function of pH, was determined by using 100 mM buffer at pH ranging from 3 to 10 using 10 mM dopamine as a substrate. pH stability was determined by pre-incubating the enzyme in 100 mM buffer (pH 3–10) for 1 h at room temp. PPO activity was assayed as described above, with dopamine as the substrate. Acetate, phosphate and Tris–HCl buffers (100 mM) were used for the pH ranges of 3–6, 5–8 and 7–10, respectively.

3.7. Effect of temperature on PPO activity

The temperature optimum of PPO was screened at various temperatures ranging from 20 to 60 °C. The thermal stability of PPO was tested at various temperatures ranging from 20 to 80 °C. The PPO was pre-incubated at the indicated temperature for 30 min and adjusted back to 4 °C before assaying using 10 mM dopamine as the substrate.

3.8. Effect of inhibitors on latex PPO activity

The effects of several inhibitors (4-hexylresorcinol, resorcinol, ascorbic acid, sodium metabisulphite, dithiothreitol, thiourea, sodium azide, 2-mercaptoethanol and sodium chloride) on latex PPO activity were determined in reaction mixtures containing 10 mM dopamine, various inhibitors as indicated (Table 6) and 20 μg enzyme taken from resuspended acetone ppt solution.

3.9. Effect of detergents on latex PPO activity

The effect of various detergents on latex PPO activity obtained after dialysis of B-serum was determined. Anionic (SDS and DOC), cationic (CTAB) and non-ionic (Triton X-100 and Tween 20) detergents were used. Enzyme assays were conducted as above.

3.10. Polyacrylamide gel electrophoresis and isoelectric focusing

SDS-PAGE was performed by method of Laemmli (1970). Standard protein markers were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and ∞-lactalbumin (14 kDa).

Isoelectric focusing was performed on 5% polyacrylamide gel with 2% Biolyte 3/10 ampholytes in Bio-Rad minigel IEF apparatus (Model 111 Mini IEF Cell). The potential difference was increased stepwise according to the manufacturer's instructions. The PPO activity bands were stained by incubating or dipping gel in the PPO assay solution containing 10 mM dopamine.

3.11. Protein determination

Protein concentration was determined by method of Lowry et al. (1951), with bovine serum albumin as standard.

Acknowledgements

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