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Kinetics and Thermal Stability of Two Peroxidase Isozymes From *Eupatorium odoratum*

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Abstract

The *Eupatorium odoratum* leaf peroxidase exists as at least seven distinct isozymes (three cationic, three anionic, and one neutral). These isozymes were identified and separated by preparative iso-electric focusing. Thermal stability, including the activation enthalpy (ΔH^*), free energy of inactivation (ΔG^*) and activation entropy (ΔS^*), and kinetic studies of two isozymes, one having a pI of 5.0 (E5) and another one having a pI of 7.0 (E7) with mol mass of 43 and 50 kD, respectively, were studied in detail. Of the molecular weight of E5 and E7, 25 and 32% correspond to the carbohydrate content of the isozymes. Optimal pH was in the acidic range of 3.6–3.8 for E5 and 3.8 for E7 with the oxidation of ABTS. E7 and E5 showed activation energy for inactivation, 194.8 and 145.4 kJ/mol, respectively. Both the isozymes showed distinct substrate specificity. The catalytic specificity constant for E5 and E7 were 112×10^5 and 124×10^5/s·*M*, respectively, when 2,2'-azino-*bis*-(3-ethylbenz-thiazoline-6 sulfonic acid) was used as the substrate. Maximum affinity (i.e., lowest K_m value) to H_2O_2 was shown by E5 and E7 along with Pyrogallol and was 0.02 and 0.05/s·*M*, respectively.

Index Entries: Arrhenius equation; *Eupatorium odoratum*; kinetics; peroxidase; thermal stability.

Introduction

Peroxidase (EC 1.11.1.7; donor: hydrogen peroxide oxidoreductase) catalyses the oxidation of various electron donor substrates in the presence of hydrogen peroxide and exhibits a wide range of substrate specificity.

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Three classes of peroxidases have been identified. Class I comprises the intracellular peroxidases including cytochrome-*c* peroxidase, ascorbate peroxidase, and the gene-duplicated bacterial catalase-peroxidases. Class II contains the secretory fungal enzymes such as manganese peroxidase and lignin peroxidase. Class III consists of secretory plant peroxidase. This enzyme is utilized in microanalysis *(1)* and in organic synthesis for the production of polymers and for the biotransformation of various drugs and chemicals *(2)*. It is reported to be potent for the construction of a cheap time temperature indicator device *(3)*. Conducting polymers such as polyaniline can be synthesized in the presence of peroxidase, hydrogen peroxide, and sulfonated polystyrene and poly(vinyl-phosphonic acid) as polymeric templates at acidic pH. Other major applications of the enzyme include its use in enzymatic determination of phenols *(4)* and in dye degradation *(5,6)*. There is a need to identify an alternate source of peroxidase with good kinetic properties and thermal stability for replacing enzymes obtained from horseradish and soybean, the sources of commercial plant peroxidases, cultivated and harvested in countries with relatively cool climates. The enzyme cost can be reduced either by reducing the production cost and/or by extracting it from cheaply available plant sources. *Eupatorium odoratum* (Family–Asteraceae) is a plant of common occurrence popularly known as "siam weed" and is considered a menace to the ecosystem because of its rapid propagation. Easy availability of this siam weed rich in the peroxidase enzyme adds to the economic feasibility of extraction of the enzyme from its leaves. It is peroxidase that is concerned with the scavenging of H_2O_2 in leaves because chloroplasts lack catalase *(7)*. Usually peroxidase activity increases with leaf development *(8)* and the mature leaves provide more peroxidase than the younger ones. Storage and operational stability are the main prerequisites of enzymes to be used in various applications *(9)*. Hence, this article deals with purification and characterization of two peroxidase isozymes extracted from the mature leaves of *E. odoratum*, giving emphasis on the thermal stability and substrate specificity studies, in order to be used as a cheap substitute of the commercial enzyme.

Materials

ABTS [2,2'-azino-*bis*-(3-ethylbenz-thiazoline-6 sulphonic acid], guaiacol, ortho phenylene diamene (*o*-PD), acrylamide, *bis*-acrylamide, Tris hydrochloric acid, ammonium persulphate, *N,N,N',N'*-tetramethyl ethylene diamine (TEMED), and sodium dodecyl sulphate (SDS) were purchased from Sigma (St. Louis, MO). Ammonium sulphate (enzyme grade) was purchased from SISCO Laboratories, Mumbai, India. All other reagents used were of analytical grade. Fresh leaves of *E. odoratum* (Asteraceae), which grows profusely in Kerala, were procured from the campus of Regional Research Laboratory, Thiruvananthapuram, India.

Methods

Enzyme Purification

Fresh mature leaves of *Eupatorium* were homogenized with distilled water in a mortar with pestle for 5–10 min at 30 ± 2°C. The extract was filtered and centrifuged at 14,000*g* for 10 min. The enzyme was precipitated from the crude extract with 50 to 75% ammonium sulfate at 4 ± 1 °C and the precipitated enzyme was centrifuged at 14,000*g* for 10 min. The precipitate was dissolved in 0.05 *M* acetate buffer (pH 4.5) and dialyzed overnight against the same buffer. Ultrafiltration of the sample was done in Amicon ultrafiltration tubes having a nominal molecular weight cut off (NMWCO) of 10 kD. The enzyme was dialyzed against deionized water for 16 h. Preparative iso-electric focusing (PIF) was carried out using a Rotofor system (BIORAD) equipped with a water recirculation chiller and vacuum pump (1% Biolyte Ampholyte from BIORAD of 3.0/10.0 pH). Twenty fractions were collected from the chambers and two enzyme fractions, one separated at pI 5.0 (E5) and another one at pI 7.0 (E7) (43 and 50 kD, respectively), were selected for further studies.

Electrophoresis

Both Native and SDS-polyacrylamide gel electrophoresis (PAGE) were performed. The separating acrylamide gel was 12% with 1-mm thickness and stacking gel was 5%. Electrophoresis was carried out at 200 V and 110 mA. Coomassie blue staining was done.

Estimation of Carbohydrate

The degree of glycosylation was estimated by the phenol-sulfuric acid method with galactose as standard *(10)*. To 1 mL of the sample, 30 mL of 80% (w/v) phenol solution were added followed by 2.5 mL of concentrated H_2SO_4. The color of the solution was measured at 490 nm in a spectrophotometer.

Peroxidase Assay Procedures Using Different Substrates

Assay of peroxidase was performed with substrates like ABTS, Guaiacol, *o*-PD, and Pyrogallol.

ABTS as substrate *(11)*: Peroxidase activity toward ABTS was measured by monitoring the absorbance increase at 405 nm in mixture containing 0.36 m*M* ABTS and 5 m*M* H_2O_2 in 0.05 *M* acetate buffer, pH 5.0 (e_{405} = 36.8/m*M*·cm).

o-PD as substrate *(12)*: Enzyme activity was assayed with 5 m*M* of *o*-PD and 0.5 m*M* H_2O_2 in citrate buffer (0.1 *M*; pH 4.5) and the absorbance increase caused by *o*-PD oxidation was monitored at 450 nm (e_{450} = 1.05/mM·cm).

Pyrogallol as substrate *(13)*: Reaction mixture contained 5.5 m*M* Pyrogallol and 4 m*M* H_2O_2 in citrate-phosphate buffer (0.025 *M*; pH 6.0).

The absorbance increase caused by pyrogallol oxidation was monitored at 420 nm (e_{420} = 2640/*M*·cm).

Guaiacol as substrate *(14)*: The peroxidase activity of the enzyme was monitored by the oxidation of guaiacol to tetraguaiacol at 470 nm (e_{470} = 26.6/*M*·cm). The reaction mixture contains 60 m*M* phosphate buffer (pH 6.1), 1 m*M* H_2O_2, and 16 m*M* substrate.

Protein estimation was done by Lowrys' method *(15)* and specific activity of the enzyme was calculated as unit activity per milligram of protein. In all the substrates, 1 U of peroxidase activity represents the amount of enzyme catalyzing the oxidation of 1 mmol of the substrate in 1 min.

pH Optimum and Stability for Enzyme Production

pH optima were determined by incubating the enzymes for 20 min in appropriate buffer (pH 3.0 to 6.0 acetate buffer, pH 6.0 to 8.0 phosphate buffer). Stability was also determined at different pH (3.6, 4.0, and 5.0) in the corresponding buffer for definite periods of time.

Thermal Stability and Activation Energy Calculations

The peroxidase isozymes E5 and E7 were exposed to different temperatures ranging from 20 to 90°C to study the optimum temperature for the enzyme activity. Activation energy for thermal inactivation (*Ea*) of E5 and E7 was determined by plotting the inactivation rate constant (*kr*) as a function of temperature, in the range 40 to 70°C (*kr* was calculated from the slopes by plotting logarithmic percentage of residual activity vs time). Activation enthalpy (ΔH^*) was calculated from the equation $\Delta H^* = Ea - RT$, (*R* is universal gas constant and *T* is the absolute temperature). The values for free energy of inactivation (ΔG^*) at different temperatures were obtained from the equation $\Delta G^* = -RT \ln(krh/kT)$ where *h* is the Planck constant and *k* is the Boltzmann constant. Activation entropy (ΔS^*) was calculated from $\Delta S^* = (\Delta H^* - \Delta G^*)/T$.

Kinetic Studies

The rates of oxidation of different reducing substrates like ABTS, guaiacol, *o*-PD, and pyrogallol were determined in the presence of H_2O_2 at various concentrations. The concentration of hydrogen peroxide used were 0.02, 0.05, 0.1, and 0.2 m*M* at constant concentrations of ABTS (0.035, 0.125, 0.25, or 0.5 m*M*), *o*-PD (2, 4, 5, and 6 m*M*), guaiacol (1.6, 2.5, 5, and 7.5 m*M*), and pyrogallol (5, 7, 9, and 11 m*M*). That is, at each substrate concentration, the hydrogen peroxide concentration was varied over the range specified. When the substrate concentration was plotted against the ratio of the substrate concentration to the rate of the reaction (Hanes-Wolf plot), the primary plot was obtained (primary Lineweaver-Burk plot showed charac-

teristic parallel lines) and true kinetic parameters of K_m (Michaelis-Menten constant) and V_{max} (velocity maximum) were obtained automatically from the secondary plot values using Sigma plot (7.01 version) 2001 software. From these values, turnover number (k_{cat}) and specificity constant (k_{cat}/K_m) were calculated. All the experiments were done at the standard assay procedure conditions and were done in triplicate.

Results and Discussion

Eupatorium leaves show an enzyme activity of 4335 IU/g of fresh leaf (using ABTS as substrate). E5 (isozyme separated at pI 5.0) and E7 (separated at pI 7.0) showed specific activities of 8987 and 8144 IU/mg of protein respectively where as the crude extract showed a specific activity of 62 IU/mg (Table 1). Although the enzyme showed seven isozymes, only two were selected, owing to its high specific activity and one being acidic (separated at pI 5.0, shown as E5) and the other being neutral (E7). The molecular mass of E5 and E7 were 43 and 50 kD, respectively (Fig. 1). The difference in molecular weight can be attributed to changes in carbohydrate content of the isozymes. Phenol-sulphuric acid analysis for carbohydrates revealed that 25 and 32% of the mol wt of E5 and E7 corresponds to the carbohydrate content of the isozymes.

The isozymes showed an optimal pH in the acidic range of 3.6–3.8 for E5 and a narrow pH optimum of 3.8 for E7 with the oxidation of ABTS. Although the enzymes showed better activity at low pH, the stability was better in the higher pH range. Similar results regarding pH have been reported in marula fruit by Mdluli *(16)*. The activity of E5 was completely lost at pH 3.6 after a period of 3 h. E7 retained its activity for 4 h of incubation at pH 5.0 (Fig. 2).

Optimum temperatures for the catalytic activity of E5 and E7 were found to be 40 and 30°C, respectively. The activity of E5 was rapidly lost above 65°C, whereas E7 enzyme retained some activity even at 75°C. E7 showed better thermal stability than E5 (Figs. 3 and 4) and showed a half-life of almost 2 h even at 70°C. Energy for inactivation *Ea* was calculated from the Arrhenius plot (Fig. 5) and was found to be 145.4 kJ and 194.8 kJ/mol, respectively for E5 and E7. High activation energy for inactivation (i.e., the energy required to denature the enzyme) of E7 stresses the heat stability of the said isozyme over the other (Table 2). Heat-resistant peroxidase isoenzymes are reported to have activation energies for inactivation of 104 kJ/mol for carrot *(17)*, 194.1 kJ/mol for cauliflower *(18)*, and 142.3 kJ/mol for horseradish *(19)*. Thermal stability of class III plant peroxidases is attributed to glycosylation *(20)* and the deglycosylated peroxidases, which usually shows low stability *(21)*. Generally high thermostability of peroxidases and lipoxigenases is attributed to the presence of sugars in their structure *(22)*. The difference in the inactivation energy *Ea* as well as activation entropy, ΔS^* of the isozymes can be attributed to higher glycosylation level of E7 (32%) compared to E5 (25%). Thermal parameters

Table 1
Purification of the Peroxidase From *Eupatorium* Leaves

Sample	Total protein (mg)	Total enzyme activity (IU)	Specific activity (IU/mg)	Activity yield (%)	Purification fold
Crude	1509	93,558	62	100	1
Ammonium sulfate ppt.	140.7	91,005	647	97	10.4
After ultrafiltration	29	57,898	1996	62	32
E5	1.97	17,704	8987	19	145
E7	2.25	18,324	8144	20	131

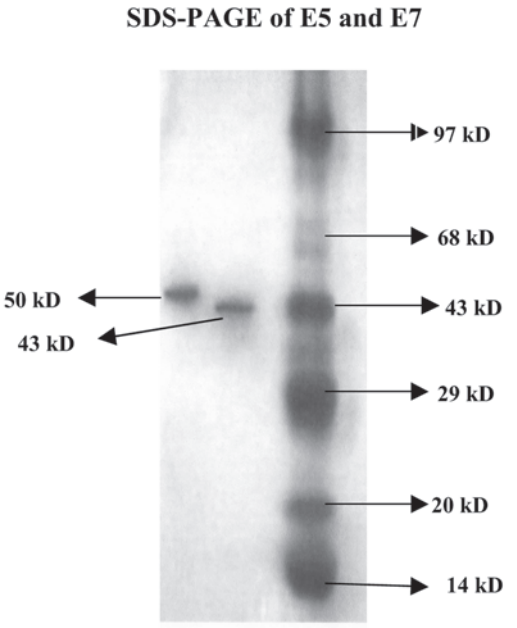

Fig. 1. SDS-PAGE of E5 and E7. Lane 1, E7; lane 2, E5; lane 3, molecular markers.

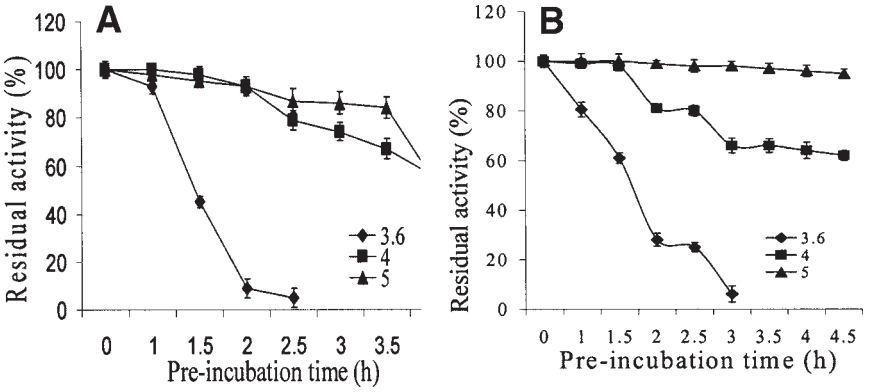

Fig. 2. pH stability of E5 **(A)** and E7 **(B)**.

are reported to depend on slight variations in the amino acid composition and molecular structure of the enzyme (including the degree of glycosylation) *(23)*.

The presence of ping-pong kinetics (figures not shown) is consistent with the typical oxidation of peroxidases by H_2O_2, followed by oxidation of the aromatic reductant. The isozymes displayed parallel lines for the primary Lineweaver-Burk plot within the specified concentration of both hydrogen peroxide and the reducing substrate, above which, enzyme inhi-

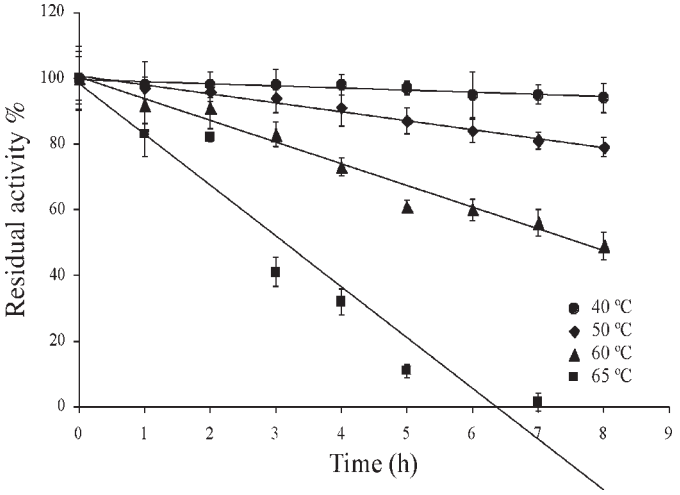

Fig. 3. Thermal stability of E5. Thermal stability of E5 was determined by incubating at 40, 50, 60, and 65°C and the residual activity percentage was monitored after every hour using ABTS assay (each experiment was repeated three times with three samples each). pH 5.0, 5 m*M* H_2O_2, 0.36 m*M* ABTS, and 100 mL of enzyme (100% = 59 IU/mL).

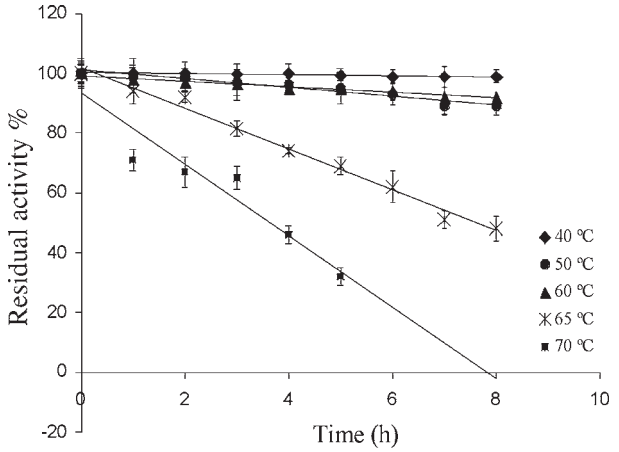

Fig. 4. Thermal stability of E7. Thermal stability of E7 was determined by incubating at 40, 50, 60, 65, and 70°C and the residual activity percentage was monitored after every hour using ABTS assay (each experiment was done in triplicate). pH 5.0, 5 m*M* H_2O_2, 0.36 m*M* ABTS, and 100 mL of enzyme (100% = 62 IU/mL).

bition occurred resulting from the substrate inhibition combined with oxidation of the iron at the haem group, forming oxyperoxidase *(24)*. The kinetic constants of the enzyme-catalyzed oxidation of ABTS and reduction of H_2O_2 are explained in Table 3. Hydrogen peroxide is a suicidal substrate for peroxidases *(25,26)*. The second (reducing) substrate such as ABTS, *o*-PD, guaiacol, or pyrogallol protects the peroxidase active site from the inactivating action of H_2O_2. Hence, for each substrate, we considered the

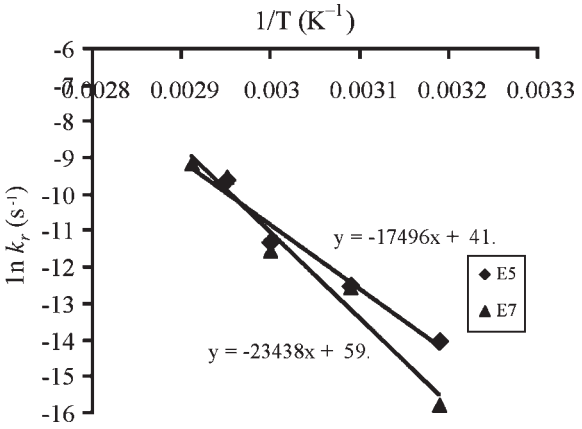

Fig. 5. Arrhenius plot for thermal inactivation of isozymes, E5 and E7. The activation energy (*Ea*) was obtained from the slope of the plot. (Inactivation rate constant, *kr*, was calculated from the slopes by plotting logarithmic percentage of residual activity vs time.)

Table 2
Thermal Inactivation Parameters of *Eupatorium* Peroxidase Isozymes[a]

Incubation temperature (K)	Half-life (h)	Inactivation rate constant (*kr*)	ΔG^* (kJ/mol)	ΔH^* (kJ/mol)	ΔS^* (J/mol ·K)
Isozyme E5 (Inactivation energy, *Ea* = 145.4 kJ/mol)					
313	239.1	8.1×10^{-7}	113.3	142.8	94.2
323	52.2	3.7×10^{-6}	112.9	142.7	92.2
333	15.8	1.2×10^{-5}	112.2	142.6	88.4
338	2.8	6.9×10^{-5}	110.1	142.6	96.2
Isozyme E7 (Inactivation energy, *Ea* = 194.8 kJ/mol)					
313	1394.9	1.4×10^{-7}	117.9	192.2	237.3
323	53.3	3.6×10^{-6}	112.9	192.1	251.1
333	19.1	1.0×10^{-6}	113.7	192.0	235.1
338	2.6	7.4×10^{-5}	109.9	191.9	242.9
343	1.8	1.1×10^{-4}	110.5	191.9	237.4

[a]at pH 5.0, 5 m*M* H_2O_2, 0.36 m*M* ABTS, and 100 mL of enzyme.

concentration of both H_2O_2 and the reducing substrate (ping-pong kinetics) to optimize the enzyme activity. Specificity constant k_{cat}/K_m provides an index to describe the catalytic efficiency of the enzyme for the competing substrates. ABTS was found to be the best substrate for both the isozymes owing to high k_{cat}/K_m. Maximum affinity of H_2O_2 (lowest K_m value) was observed with pyrogallol for both the isozymes and it was 0.02 and 0.05 m*M*, respectively, for E5 and E7. When ABTS was used as the reducing substrate, the K_m value of E5 and E7 for H_2O_2 was 0.35 and 0.43 m*M*, respec-

Table 3
Kinetic Constants of the Enzyme for the Catalysis of the Reducing Substrates and H_2O_2[a]

K_m of reducing substrates (mM)	K_m of H_2O_2 (mM)	V_{max} (mM/min ·mg)	k_{cat} (s^{-1})	k_{cat}/K_m of the reducing substrate (s^{-1} M^{-1})	k_{cat}/K_m of H_2O_2 (s^{-1} M^{-1})
Isozyme E5 (molecular weight = 43 kD)					
ABTS (0.21)	0.35	3326	2383	112×10^5	677×10^4
Guaiacol[b]	–	–	–	–	–
o-PDA (9)	0.28	1039	744.6	827×10^2	267×10^4
Pyrogallol (55.4)	0.016	143	102.5	1849	632×10^4
Isozyme E7 (molecular weight = 50 kD)					
ABTS (0.23)	0.43	3400	2833	124×10^5	657×10^4
Guaiacol (28.6)	0.34	3013.5	2511.7	878×10^2	735×10^4
o-PD[b]	–	–	–	–	–
Pyrogallol (2.3)	0.05	1470	1225	535×10^3	232×10^5

[a]At pH 5.0, temperature 30 ± 2°C, 100 mL of enzyme. Concentration ranges: 0.035–0.2 mM ABTS , 5–11 mM pyrogallol, 2–6 mM *o*-PD, 1.6–10 mM guaiacol, and 0.02–0.2 mM H_2O_2.
[b]Negligible activity.

tively. K_m values for H_2O_2 for some other plant peroxidases reported are, 1.77 m*M* (16), 0.85 m*M (27)*, and 1.5 m*M (28)*. E5 catalyzed the oxidation of *o*-PD, although the specificity constant was much less because of the low turnover number (k_{cat}) compared with that of ABTS, whereas E7 showed an activity too negligible to be considered. The isozyme E7 was found to be a better catalyst than E5 for the oxidation of pyrogallol because of the low K_m value. Similarly, another substrate, guaiacol was oxidized well by the neutral isozyme, E7 whereas negligible activity was observed with the anionic isozyme under the specified conditions. It has been reported earlier that the activity of some anionic peroxidases depend on even the type as well the concentration of buffer *(13)*. Anionic peroxidases have also been reported to be poorer biocatalysts for the oxidation of guaiacol *(29)*. The specificity of E5 to the reducing substrates was in the order, ABTS > *o*-PD > pyrogallol and for E7 it was ABTS > pyrogallol > guaiacol. Efficiency of *Eupatorium* peroxidase catalysis depends on the chemical nature of the substrates. The slight difference in the substrate specificity of the isozymes may be the result of the differences in the active site conformation, structural stability and compactness in addition to the degree of glycosylation, although more comprehensive structural analysis will be needed to state the reason.

The lyophilized peroxidase remained stable and active without any loss of activity for more than 2 yr, which is as good as commercial Sigma peroxidase preparation.

Eupatorium peroxidase can become a very cost-effective alternative for the other known commercial peroxidases because of its abundance, easy propagation, and wide distribution. A new peroxidase like this with wide substrate specificity and good thermal stability could be of interest for the development of new electrochemical biosensors for bioanalytical applications *(29,30)*, medical diagnostics, and biotransformations.

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