Research Article

Luffa aegyptiaca (Gourd) Fruit Juice as a Source of Peroxidase

R. S. S. Yadav,[1] K. S. Yadav,[1] and H. S. Yadav[2]

[1] *Department of Chemistry, D.D.U. Gorakhpur University, Gorakhpur 273 009, India*
[2] *Department of Chemistry, NERIST, Nirjuli, 791109, India*

Correspondence should be addressed to R. S. S. Yadav, rssy_chemistry@rediffmail.com

Peroxidases have turned out to be potential biocatalyst for a variety of organic reactions. The research work reported in this communication was done with the objective of finding a convenient rich source of peroxidase which could be used as a biocatalyst for organic synthetic reactions. The studies made have shown that *Luffa aegyptiaca* (gourd) fruit juice contains peroxidase activity of the order of 180 enzyme unit/mL. The K_m values of this peroxidase for the substrates guaiacol and hydrogen peroxide were 2.0 and 0.2 mM, respectively. The pH and temperature optima were 6.5 and 60°C, respectively. Like other peroxidases, it followed double displacement type mechanism. Sodium azide inhibited the enzyme competitively with K_i value of 3.35 mM.

1. Introduction

Peroxidase (E.C. 1.11.1.7) is a heme-containing enzyme, which catalyses the oxidation of a wide variety of organic and inorganic substrates using hydrogen peroxide as the electron acceptor [1, 2]. Peroxidases are widely distributed in living organisms including microorganisms, plants, and animals [3]. It is mainly located in the cell wall [4], and it is one of the key enzymes controlling plant growth and development. It is involved in various cellular processes including construction, rigidification, and eventual lignifications of cell walls [5], suberization [6], organogenesis [7], phenol oxidation [8], crosslinking of cell wall proteins [9], and protection of tissue from damage and infection by pathogenic microorganisms [10–12]. It is also used in clinical diagnosis and microanalytical immunoassays because of its high sensitivity. Apart from these applications, peroxidases have been used for biotransformations in organic synthesis [13].

Recent studies have revealed that not all peroxidases are similar in their structures and functions [14–21]. Lignin peroxidase differs from horseradish peroxidase in the sense that lignin peroxidase directly oxidizes veratryl alcohol whereas horseradish peroxidase cannot [17]. Soyabean peroxidase [17] has lignin peroxidase type activity, but it is more stable at acidic pH and at higher temperatures than the lignin peroxidase. During last decades, peroxidases have turned out to be potential biocatalysts [22] for variety of organic reactions, for example, enantioselective reduction of hydroperoxides [23], hydroxylation of arenes [24], epoxidation of olefins [20], halogenation [25], N-oxidation [26], and sulfoxidation [27]. The studies reported in this communication were performed with the objective of finding a convenient rich source of peroxidase which could be tested for the above organic biotransformations. We have found that *aegyptiaca* fruit juice is a novel good source of peroxidase. The enzymatic properties of peroxidase of *L. aegyptiaca* fruit juice have been reported in this communication.

2. Materials and Methods

2.1. Chemicals. Guaiacol was from Sigma Chemical Company, St. Louis USA. All other chemicals used in these investigations were either from Himedia Laboratory Ltd, Mumbai or from E. Merck (India) Ltd., Mumbai, and were used without further purifications.

2.2. Isolation of the Enzyme. The enzyme was isolated by cutting the *L. aegyptiaca* fruit into the small pieces, crushing the pieces in mortar with pestle, and extracting the juice by keeping the pieces in four layers of cheese cloth and squeezing it. The juice was centrifuged using Sigma (Germany) model 3 K-30 refrigerated centrifuge at 4000 g for 20 min at 4°C to remove the cloudiness of the juice. The clear juice was

stored at 4°C. The enzyme stored in this way has reasonable activity even after 3 months.

2.3. Peroxidase Activity Assay.

Peroxidase activity of the enzyme was measured in 1 mL reaction solution containing 50 mM sodium phosphate buffer pH 7.0 at 30°C using 5 mM guaiacol, 0.6 mM hydrogen peroxide as the substrates and by monitoring the absorbance changes at $\lambda = 470$ nm using molar extinction coefficient value of $2.66 \times 10^4 \, M^{-1} \, cm^{-1}$ for the product tetraguaiacol formed by the enzymatic reaction [28, 29]. The reaction solution was allowed for thermal equilibration for 10 minutes, $10 \, \mu L$ of the enzyme stock 180 IU/mL was added, and activity measurement was started immediately and was continued for 180 seconds. All spectrophotometric measurements were done with UV/Vis spectrophotometer Hitachi (Japan) model U-2000 which was fitted with electronic temperature control unit for variation of temperature in the cuvettes. The least count of the absorbance measurement was 0.001, and one enzyme unit is the amount of enzyme which produces $1 \, \mu mol/min$ of the product.

2.4. Kinetic Studies.

The steady state kinetics of the enzyme was studied using guaiacol and H_2O_2 as the variable substrates and monitoring the steady state formation of tetraguaiacol as mentioned in the assay section. While varying the concentration of guaiacol, the concentration of H_2O_2 was fixed at enzyme saturating value 0.6 mM. Similarly, while varying the concentration of H_2O_2, the concentration of guaiacol was fixed at the enzyme saturating value 5 mM. The K_m values were calculated using linear regression analysis of the data points of double-reciprocal plots. Each point of steady state velocity was an average of triplicate measurements, and the percentage standard deviation was less than 5%.

The pH optimum was determined by measuring the relative activity of the enzyme in the pH range 2.5–9.0 using buffer prepared with $H_3PO_4/NaH_2PO_4/Na_2HPO_4$. The composition of the reaction solution was the same as mentioned in the peroxidase activity assay. The temperature optimum was determined by measuring the relative activity of the enzyme in the temperature range 20–80°C at fixed pH 7.0 and using the reaction solution of the composition mentioned in peroxidase activity assay. Before each measurement, the reaction mixture in the spectrophotometer cuvette was allowed for 10 min for temperature equilibration, the reaction was initiated by the addition of $10 \, \mu L$, of concentrated enzyme stock having 180 IU/mL, and the activity was measured immediately.

2.5. Inhibition Study.

The effect of sodium azide on the activity of the enzyme was studied by monitoring the steady-state velocity of the enzyme-catalysed reaction in the presence of varying concentrations of sodium azide (0–50 mM) using 5 mM guaiacol and 0.6 mM H_2O_2 in 50 mM NaH_2PO_4/Na_2HPO_4(pH 6.5) at 30°C. $10 \, \mu L$ of the enzyme stock of 180 IU/mL was added in 1 mL of the assay solution. The inhibition constant was determined by drawing double-reciprocal plots at different concentrations of the inhibitor using guaiacol as the variable substrate and then drawing the secondary plot of the slopes versus the concentrations of the inhibitor.

3. Results and Discussion

The increase of absorbance at $\lambda = 470$ nm, ΔA_{470}, due to the conversion of guaiacol to tetraguaiacol with time in a peroxidase assay solution containing *L. aegyptiaca* fruit juice, is given by the equation $\Delta A_{470} = 2.4 \times 10^{-3} \, t + 11.8 \times 10^{-3}$ which has a correlation factor of 0.9982 determined by the linear regression analysis. There is no increase in absorbance at $\lambda = 470$ nm in assay solutions containing no enzyme or the denatured enzyme which was obtained by one hour boiling in water. These results indicated that *L. aegyptiaca* fruit juice contained peroxidase activity. The analysis of the steady state kinetic measurements showed the presence of 180 IU/mL of peroxidase in the juice. Thus, *L. aegyptiaca* fruit juice is a rich and convenient source of peroxidase enzyme, and it can be used for various biotransformation reactions.

Figure 1(a) shows the Michaelis-Menten curve using guaiacol as the variable substrate at the saturating concentration of the other substrate, H_2O_2 (0.6 mM) for the determination of K_m value for guaiacol. The double-reciprocal plot shown in Figure 1(b) is linear having the correlation factor of 0.9729 showing that the enzyme obeyed Michaelis-Menten kinetics [30]. The Michaelis-Menten and double-reciprocal plots using hydrogen peroxide as the variable substrate at the fixed enzyme saturating concentration of guaiacol (5 mM) are shown in Figures 2(a) and 2(b), respectively. The correlation factor for the points on the double-reciprocal plot Figure 2(b) is 0.9864. The calculated K_m values for guaiacol and hydrogen peroxide were 2.0 mM and 0.2 mM, respectively. The corresponding values of K_m for *horseradish peroxidase* [31], *Turkish black radish* [32], and *Solanum melongena* fruit juice [29] were 0.8 mM and 0.1 mM, 36.0 μM, and 8.4 μM, and 6.5 mM and 0.33 mM, respectively. The reported enzyme has lower affinity for both the substrates than the peroxidases of *horseradish peroxidase* and *Turkish black radish* (*Raphanus sativus* L.) but higher affinity for both the substrates as compared to the peroxidase of *S. melongena* fruit juice.

Since peroxidases are known to follow double displacement type kinetics [33], *L. aegyptiaca* fruit juice peroxidase was also analysed for this type of kinetics by measuring the steady state velocity of the enzyme-catalysed reaction at three different fixed concentrations of the hydrogen peroxide and varying the concentration of guaiacol at each hydrogen peroxide concentration and also at three different fixed concentrations of guaiacol and varying the concentration of hydrogen peroxide. Double reciprocal plots (not shown here) in both cases have been found to be parallel straight lines confirming that the reported peroxidase also follows double displacement type mechanism observed in case of other peroxidases [33].

In order to use the reported peroxidase under optimal conditions of pH and temperature, the dependence of the enzyme activities on the pH and temperature of the reaction
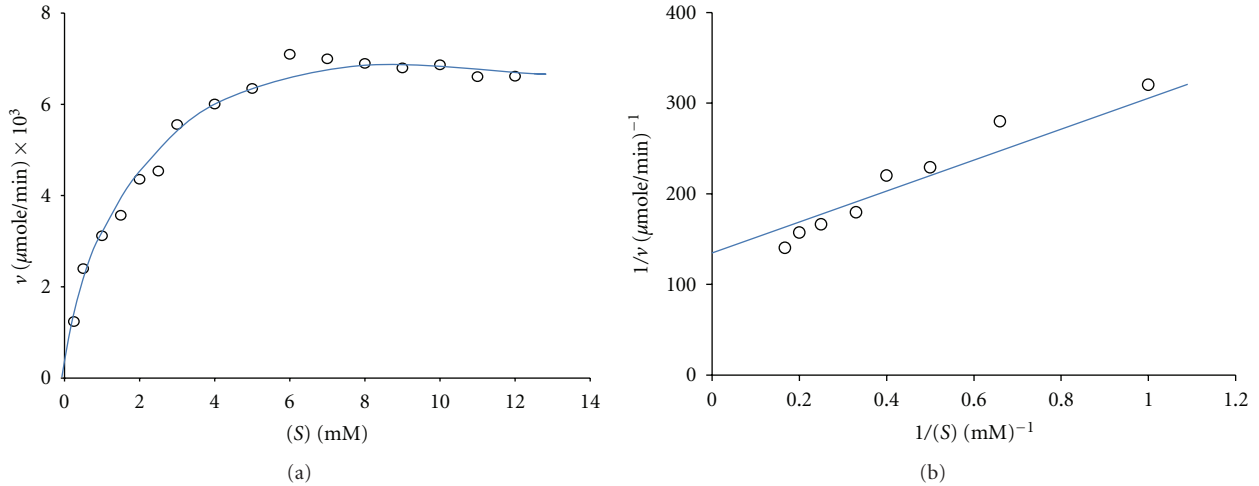
FIGURE 1: (a) Michaelis-Menten and (b) double-reciprocal plots for the peroxidase using guaiacol as the variable substrate.
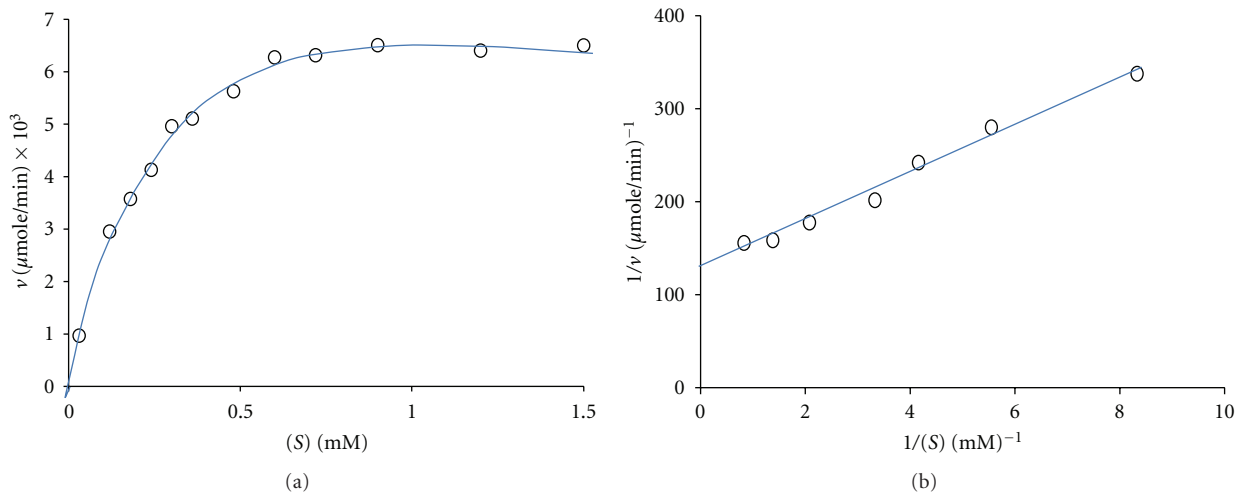

FIGURE 2: (a) Michaelis-Menten and (b) double-reciprocal plots for the peroxidase using hydrogen peroxide as the variable substrate.
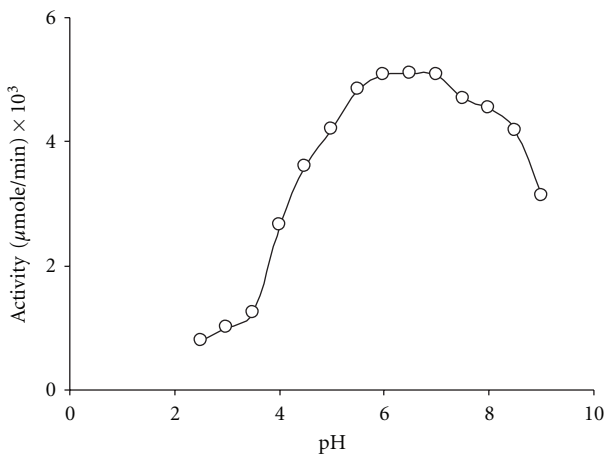

FIGURE 3: Dependence of the enzyme activity on pH of the assay solution at fixed temperature 30°C.

solutions were studied. The results are shown in Figures 3 and 4, respectively. The enzyme had pH optimum at 6.5 and showed more than half of the maximum activity in the pH range 4.0 to 9.5. Plant peroxidases of *S. melongena* fruit juice [29] and *Musa paradisiaca* stem juice [34] have been studied in our laboratory. The pH optima of the peroxidases from these sources have been found to be 5.5 and 4.5 pH units, respectively. Thus the reported peroxidase can be used effectively near neutral pH, whereas the other two peroxidases have pH optima in more acidic regions.

The temperature optimum of the reported enzyme is 60°C calculated from Figure 4. The temperature optima of the peroxidases from *S. melongena* fruit juice [29] and *M. paradisiacal* stem juice [34], the other two plant peroxidases reported from our laboratory, were 84°C and 62.5°C respectively. Thus the peroxidase from *L. aegyptiaca* fruit juice has the temperature optimum on the higher temperature side as
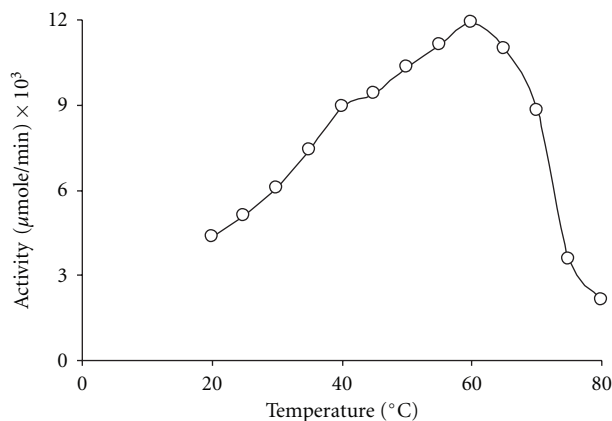
FIGURE 4: Dependence of the enzyme activity on temperature of the assay solution at fixed pH 7.0.
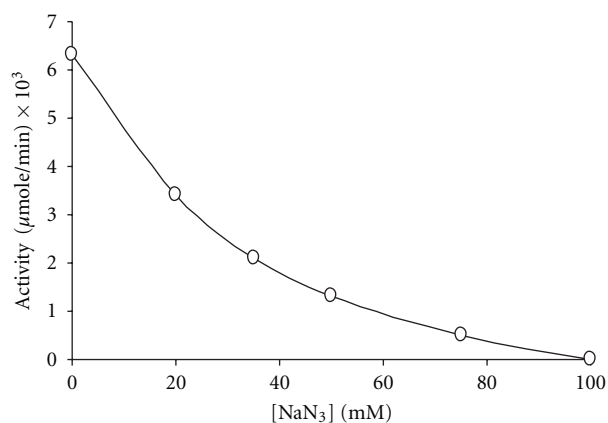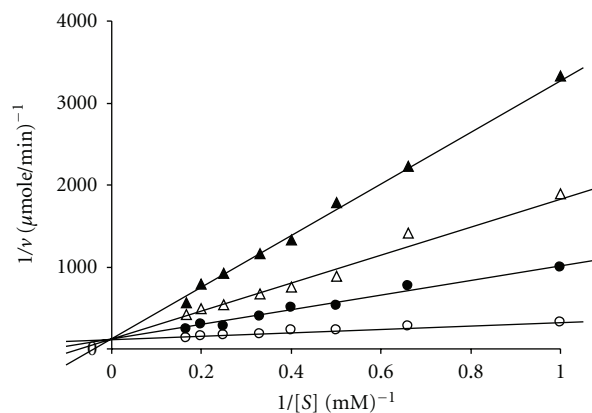

FIGURE 6: Double-reciprocal plots for peroxidase using guaiacol as the variable substrate at different concentrations of inhibitor sodium azide: ($\bigcirc$) 0.0 mM, ($\bullet$) 20.0 mM, ($\triangle$) 35 Mm, and ($\square$) 50 mM.

FIGURE 5: Inhibition of the activity of the peroxidase by sodium azide.

reactions like hydroxylation of arenes, the oxyfunctionalization of phenols and aromatic amines, the epoxidation and halogenation of olefins, the oxygenation of hetero atoms, and the enantioselective reduction of racemic hydroperoxides. This short communication reports a convenient and rich source of a peroxidase which can be used for the above transformations.

Acknowledgment

in the cases of peroxidases from *S. melongena* fruit juice [29] and *M. paradisiacal* stem juice [34].

The effect of sodium azide which is known to inhibit peroxidase activity [35] was also studied on the activity of *L. aegyptiaca* fruit juice peroxidase. The results are shown in Figure 5. The concentration of sodium azide needed to reduce the relative activity of the enzyme to half of its initial value is 20 mM. In order to decide the nature of inhibition, double-reciprocal plots were drawn in the presence of different fixed concentrations of sodium azide inhibitor and varying the concentration of guaiacol. The result is shown in Figure 6. The nature of inhibition was competitive, because the intercepts on y-axis was independent of the inhibitor concentration. The determined K_i value for sodium azide was 3.35 mM. The competitive inhibition suggests that the inhibitor competes for the binding of guaiacol substrate to the enzyme [30].

4. Conclusion

During the last decades, peroxidases have emerged as good biocatalysts for the variety of organic biotransformation

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