EXTRACTION AND KINETIC PROPERTIES OF PEROXIDASE FROM BITTER GOURD

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Abstract

Peroxidase (PODs, E.C.1.11.1.7) catalyzes the oxidation of H_2O_2 and guaiacol forming the product tetraguaiacol and water. In this study partially purified peroxidase enzyme was extracted from fresh bitter gourd by ammonium sulphate precipitation method. Guaiacol was used as a substrate for peroxidase activity determination by using UV-visible spectrophotometer. Protein content of enzyme solution was determined by Biuret method. The specific activity of peroxidase was 0.6361 U mL^{-1} and the enzyme was purified 1.99 fold over its crude extract. The optimum pH of peroxidase was 6.0 in phosphate buffer and optimum temperature was 40 ˚C. The values of K_m and V_{max} treated statistically using the linear regression method were compared with various graphical methods (Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Wilkinson). The K_m and V_{max} values of peroxidase were found to be 0.514 x10^{-2} M and 26.853 M min^{-1}, respectively, from Lineweaver-Burk plot. The reaction order for peroxidase-catalyzed reaction of conversion of guaiacol to tetraguaiacol was found to be first order. The activation energy (E_a) of peroxidase-catalyzed reaction was calculated to be 3.592 kcal mol^{-1}. The decolourisation of methyl orange (MO) by crude peroxidase from bitter gourd was studied by using spectrophotometric method.

Keywords: Bitter gourd, peroxidase, guaiacol, ammonium sulphate precipitation method, methyl orange

Introduction

Peroxidase (POD) is an oxidoreductase that catalyzes a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atoms. Peroxidases have been discovered in various plants and prokaryotic and eukaryotic microbes as well as in mammalian cells. The best characterized plant peroxidase is from horseradish, from which more than 40 isoenzymes have been isolated. In plants, peroxidases have various physiological roles in for example, degradation and synthesis of lignin in cell walls, in the defence mechanism and in cell damage (Vance *et al.,* 1980).

Commercially available peroxidase is widely employed for removal of phenols and amines from industrial wastewater, bleaching of industrial dyestuffs, lignin degradation, fuel and chemical production from wood pulp and in various organic syntheses. Peroxidase act on the removal of hydrogen atom most usually from the alcohol groups, which are combined with hydrogen peroxide in order to form molecules of water and oxidized phenolic compounds, acting as detoxifying enzymes and as a cell wall crossing linked enzyme during wounding stress (Passardi *et al*., 2005).

Several investigations have reported the use of peroxidases for the removal of azo dyes in aqueous phase, obtaining percentages of decolourization ranging from 50 to 90 % using different operating conditions (Nouren and Nawaz, 2015). In the literature several studies employing soybean peroxidases (SBP) for the decolouration of azo dyes can be found.

The aim of this research was to study the extraction and biochemical characterization of peroxidase from bitter gourd.

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Materials and Methods

Sample Collection

Bitter gourd samples were purchased from local shop, Kyeemyindaing Township, Yangon Region. Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich, England. All other chemicals used were of analytical reagent grade. In all investigations, the recommended standard methods and techniques involving both conventional and modern methods were provided.

Solution Preparation

Guaiacol (0.12 mL) was dissolved in phosphate buffer (pH 7) and the volume made up to the mark in a 50 mL volumetric flask. Hydrogen peroxide (0.128 mL of 50 % v/v) was dissolved in phosphate buffer (pH 7) and the volume made up to the mark in a 100 mL volumetric flask. Bovine Serum Albumin 0.2 g was dissolved in a small amount of distilled water, then 1mL of 10 % sodium hydroxide was added and the volume made up 20 mL with distilled water.

Extraction and Partial Purification of Peroxidase from Bitter Gourd

The fresh bitter gourd was washed with tap water, chopped and mixed with phosphate buffer pH 7.0 solution. It was stirred in ice for 2 h and filtered. Solid ammonium sulphate was slowly added to this extract to obtain 20 % first and then 70 % saturation and stirred for 2h in an ice bath (Koktepea *et al*., 2017). After standing overnight, the precipitated protein containing peroxidase enzyme was collected by centrifugation at 5000 rpm for 30 min.

Peroxidase Assay, Protein Determination and Kinetic Studies of Peroxidase from Bitter Gourd

For enzyme assay the peroxidase activity of bitter gourd was determined by the spectrophotometric method at 470 nm using guaiacol as a substrate in the presence of hydrogen peroxide (Koktepea *et al*.,2017). Substrates were mixed with different concentrations of enzyme and then the absorbance values were observed at 470 nm. Peroxidase activity assay was based on the measurement of tetraguaiacol formation in the presence of guaiacol and H_2O_2 within 2 min. The final mixture contained 0.1 mL enzyme sample, 20 mM guaiacol (1.0 mL), 22.5 mM H_2O_2 (0.1 mL), and 2.7 mL phosphate buffer (0.1 M, pH 6.0).

Protein content was determined by Biuret method using Bovine Serum Albumin (BSA) as standard at 550 nm. In brief, 4 mL of Biuret reagent solution was added to 1 mL of protein solution in a test tube. The solution mixture was stored at room temperature for 30 min and the absorbance measured (Stoscheck, 1990).

Effects of enzyme concentration, pH, temperature, reaction time and substrate concentration were studied by spectrophotometric method. The enzyme kinetic parameters of K_m, V_{max}, activation energy, and reaction order of peroxidase-catalyzed reaction were determined by spectrophotometric method.

Decolourization of Azo Dye by Peroxidase

The decolourization of methyl orange (MO) by partially purified peroxidase from bitter gourd was studied by using spectrophotometric method. Methyl orange (20 ppm) was incubated with bitter gourd peroxidase in 0.1 M citrate buffer, pH 4.0 at 30 ^oC in the presence of 2 mM hydrogen peroxide for 4 h (Ambatkar and Mukundan, 2015). Dye decolourization was monitored at 464 nm and the percent decolourization was calculated by taking untreated dye solution as control 100 % (Verma and Madamwar, 2002).

Results and Discussion

Effect of Enzyme Concentration on Peroxidase-catalyzed Reaction

Increasing the amount of enzyme increases the frequency of with which the enzyme and substrate collide. As a result, enzyme-substrate complexes form more quickly and the rate of reaction increases. The validity of enzyme assay method was tested using different concentrations of enzyme.

In this experiment guaiacol was used as substrate. Oxidized guaiacol (yellowish-brown colour) was observed (Figure 1). The absorbance of the oxidized guaiacol was found to have a linear relationship with different enzyme concentration ranging between 11.94 to 59.70 mg/mL of enzyme (Table 1 and Figure 2). The velocity of an enzymatic reactions was found to vary directly with the enzyme concentration, i.e., the more the enzyme, the faster the reaction. If the amount of is doubled the reaction rate will also be doubled generally.

(a) (b) (c) (d) (e) (f)

Figure 1 Mixture of 20 mM guaiacol and 22 mM H_2O_2 solutions (a) without enzyme (control) and with (b) 11.94 mg mL^{-1}, (c) 23.88 mg mL^{-1}, (d) 35.82mg mL^{-1} (e) 47.76 mg mL^{-1} and (f) 59.70 mg mL^{-1} enzyme solutions

Table 1 Relationship between Absorbance and Enzyme Concentration

No	Enzyme concentration (mg mL^{-1})	Absorbance at 470 nm
1	59.70	0.865
2	47.76	0.678
3	35.82	0.502
4	23.88	0.357
5	11.94	0.193

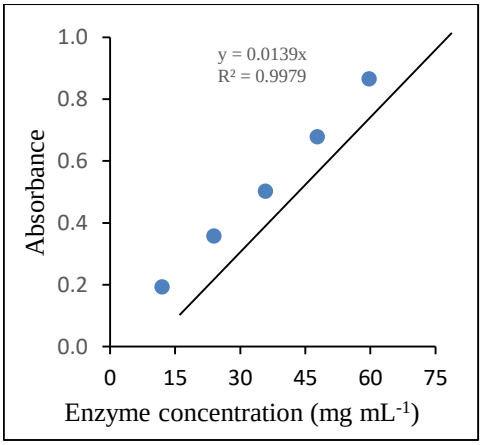

Figure 2 Plot of absorbance as a function of enzyme concentration

Calibration Curve for Protein Determination by Biuret Method

In the present work, bovine serum albumin (BSA) was used as a standard protein (Savary *et al.*, 1969). The determination of protein concentration was done using a calibration curve created using samples of known concentrations. The different absorbance values were obtained for various standard protein solutions by using a UV-visible spectrophotometer. The absorbance of protein treated with Biuret reagent was measured at 550 nm after the purple product formed.

It was found that the nature of the plot of absorbance at 550 nm *vs.* concentration of protein (mg mL^{-1}) (Table 2 and Figure 3), was a straight line passing through the origin showing that Beer's Law was obeyed.

Table 2 Relationship Between Absorbance and Concentration of Bovine Serum Albumin (BSA) Solutions

No.	Protein Concentration (mg mL^{-1})	Absorbance at 550 nm
1	2.0	0.147
2	4.0	0.283
3	6.0	0.405
4	8.0	0.524
5	10.0	0.630

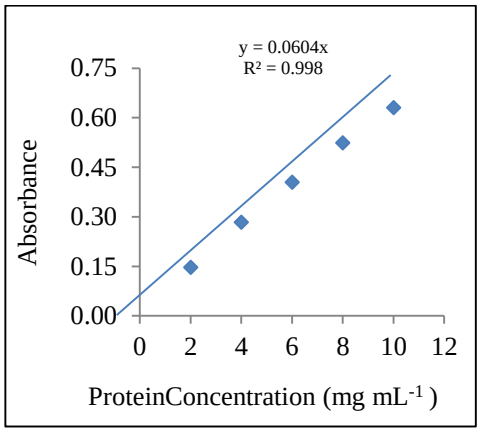

Figure 3 Calibration curve for standard protein solution

Peroxidase Activity, Protein Content and Specific Activity of Peroxidase

Enzyme activity was tested using guaiacol as substrate where the effect of peroxidase enzyme on guaiacol in the presence of hydrogen peroxide was tested (Lakshmi *et al*., 2018). Spectrophotometric method of analysis was used at a wavelength of 470 nm to quantify the activity on the basis of the production of coloured complex due to the action of the enzyme. One unit of peroxidase was defined as the amount of enzyme that caused the formation of 1 mM of tetraguaiacol per minute.

The protein content was determined by Biuret method and it was observed to be 10.8702 mg mL^{-1} (Table 3). Specific enzyme activity is the number of enzyme units per mL divided by the concentration of protein in mg mL^{-1}. The specific activity was calculated to be 0.6361 U mg^{-1}. After 70 % ammonium sulphate precipitation, peroxidase was purified to 1.99 fold over crude extract.

Table 3 Peroxidase Activity, Protein Content and Specific Activity of Bitter Gourd in Different Purification Steps

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification fold
Crude	21.9286	68.4252	0.3204	1.0
After precipitation with 20 % ammonium sulphate	31.1102	66.3363	0.4689	1.46
After precipitation with 70 % ammonium sulphate	6.9154	10.8702	0.6361	1.99

Optimum pH of Peroxidase Activity

The enzyme activity is affected by pH due to changes in the ionization pattern of ionic groups located in the lateral chains of amino acids constituents of the protein primary structure. The bell-shaped activity versus pH curve is an indication of pH influence on the enzyme molecular structure conformation (*Vitolo*, 2010).

In this work, different buffers of pH values 5.0 to 8.0 were used to determine the activity of the prepared peroxidase sample. The nature of the activity *vs.* pH curve of the enzyme (Table 4 and Figure 4) was obviously found to be unsymmetrical and the optimum pH was obtained at pH 6.0 with guaiacol as substrate.

Table 4 Relationship between Peroxidase Activity and pH of Phosphate Buffer Solution

Phosphate Buffer	pH	Peroxidase activity (U mL^{-1})
1	5.0	5.692
2	5.5	6.354
3	6.0	7.146
4	6.5	6.662
5	7.0	5.723
6	7.5	5.500
7	8.0	4.962

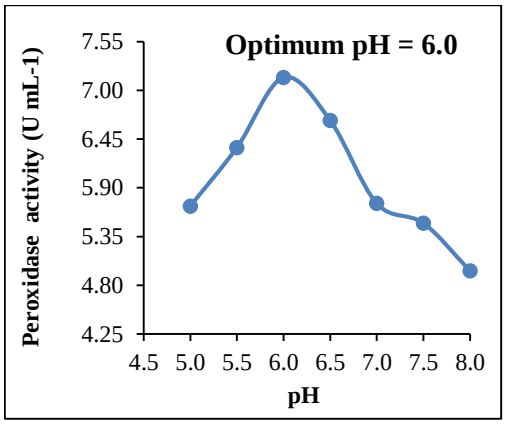

Figure 4 Plot of peroxidase as a function of pH solutions

Optimum Temperature of Peroxidase Activity

The optimum temperature of an enzyme is a temperature at which the greatest amount of substrate changes in time units (*Vitolo*, 2010). Temperature affects the **reaction rate of enzymes**, as do pH, substrate concentration and enzyme concentration. The optimum temperature for any enzyme not only changes in relation to time, but may also change in relation to changes in pH, concentration and purity of enzyme preparation.

In this study, the effect of the temperature on the peroxidase activity was investigated in the temperature range from 10 to 70 ºC. The optimum temperature for peroxidase was found to be 40 ºC in phosphate buffer pH 6.0 (Table 5 and Figure 5). The activation energy E_a of peroxidase-catalyzed reaction was calculated by Arrhenius equation, log k = -E_a/ (2.303 RT) (Whitaker, 1996). Table 6 shows the relationship between temperature and velocity of peroxidase-catalyzed reaction. Figure 6 shows the graph for determination of activation energy and Arrhenius constant. By using the constant substrate concentration throughout the experiment, rate constant (K) in Arrhenius equation can be substituted by velocity of the peroxidase-catalyzed reaction. The activation energy (E_a) was determined to be 3.592 kcal mol^{-1} from linear regression method.

Table 5 Relationship between Peroxidase Activity and Temperature of the Solution at pH 6.0

No.	Temperature (°C)	Peroxidase Activity (U mL^{-1})
1	10	2.792
2	20	3.908
3	30	5.223
4	40	6.741
5	50	5.769
6	60	5.346
7	70	3.246

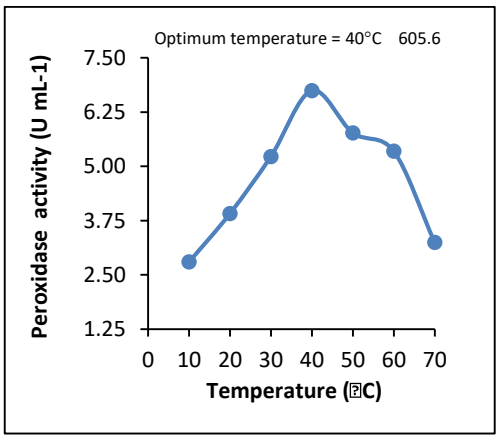

Figure 5 Plot of peroxidase activity as a function of temperature of the solutions at pH 6.0

Table 6 Relationship between Temperature and Velocity of the Peroxidase-catalyzed Reaction

Temperature (°C)	Temperature (K)	1/T (10^3 K^{-1})	Velocity (M min^{-1})	Log V
10	283	3.534	9.308	0.9689
20	293	3.413	13.026	1.1148
30	303	3.300	17.410	1.2408
40	313	3.195	22.487	1.3519

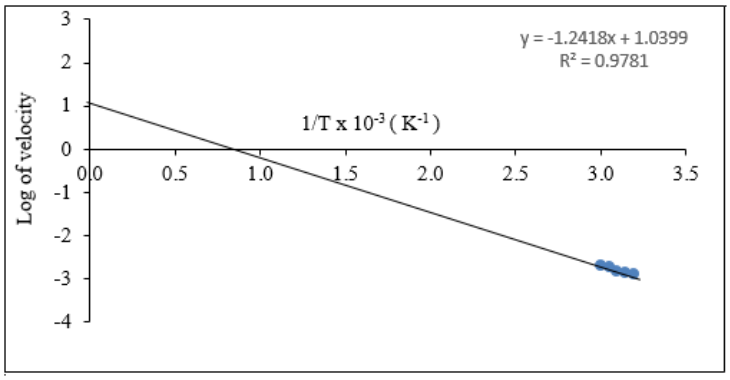

Figure 6 Plot of log of velocity as a function of 1/T for peroxidase

Effect of Reaction Time on Peroxidase-catalyzed Reaction

The activity of the enzyme is determined by the enzyme concentration, substrate concentration, pH, temperature and reaction time (Wiseman, 1985). In this work, the action of the peroxidase on guaiacol was studied in phosphate buffer of pH 6.0. The amount of tetraguaiacol liberated during the various reaction time of 1, 2, 5, 8, 11, 14, 17 and 20 min were determined by spectrophotometric method (Table 7). Figure 7 shows the plot of velocity of peroxidase reaction as a function of reaction time. At the beginning of the reaction (during 5 min), the reaction was very fast. Then, velocity decreased steadily. Therefore, in sequence studies, reaction time of 2 min was used for initial velocity measured in enzyme kinetic.

Table 7 Relationship between Reaction Time and Velocity of Peroxidase - catalyzed Reaction

No	Reaction Time (min)	Velocity (M min^{-1})
1	1	19.795
2	2	14.256
3	5	3.487
4	8	2.718
5	11	2.581
6	14	2.205
7	17	2.103
8	20	1.915

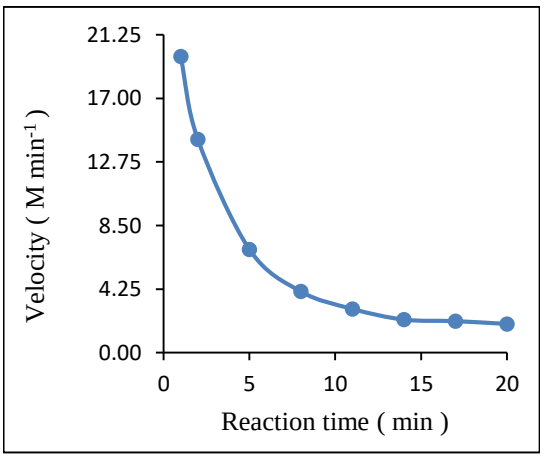

Figure 7 Plot of velocity of peroxidase-catalyzed reaction as a function of reaction time

Effect of Substrate Concentration on Peroxidase-catalyzed Reaction

The effect of substrate concentrations on peroxidase-catalyzed reaction is shown in Table 8. Each enzyme has a characteristic substrate concentration (K_m, the Michaelis-Menten constant) at which the reaction velocity is one-half maximal (Sawhney and Singh, 2000). Michaelis-Menten equation, ($V=V_{max}[S]/K_m+[S]$) explains kinetics but, because it is nonlinear, is a little hard to deal with real practical data. K_m and V_{max} were found to be 0.472 x 10^{-2} M and 23.205 M min^{-1}, respectively, from Michaelis-Menten plot (Figure 8).

Most common transform is the Lineweaver-Burk plot which is also called double reciprocal plot 1/V *vs* 1/[S]) plot. The reciprocal transformation distorts the error in the measurements as shown in Table 9 and Figure 9. The noisiest data are too heavily weighted when linear regression (Paradine and Rivert, 1970) is used to determine the best straight line. Figures 10 and 11 show Eadie-Hofstee and Hanes-Wilkinson plots, respectively, for evaluation of K_m and V_{max} values. The values of K_m and V_{max} treated statistically using the linear regression method were compared with various graphical methods. From the Lineweaver-Burk plot K_m and V_{max} value were found to be 0.515× 10^{-2} M and 26.831 M min^{-1}, respectively, by graphical method and 0.514 × 10^{-2} M and 26.853 M min^{-1} by linear regression method.

Table 8 Relationship between Substrate Concentration and Velocity of Peroxidase-Catalyzed Reaction

No	[S]×10^3 (M)	-[S]×10^3 (M)	1/[S]×10^{-3} (M^{-1})	V (M min^{-1})	1/V×10^1 (M^{-1}min)	V/[S] ×10^{-3} (min^{-1})	[S]/V×10^3 (min)
1	5	-5	0.2000	12.9487	0.7723	2.5897	0.3861
2	10	-10	0.1000	18.8462	0.5306	1.8846	0.5306
3	15	-15	0.0667	20.6923	0.4833	1.3795	0.7249
4	20	-20	0.0500	21.5128	0.4648	1.0756	0.9297
5	25	-25	0.0400	22.1282	0.4519	0.8851	1.1298
6	30	-30	0.0333	22.4359	0.4457	0.7479	1.3371
7	35	-35	0.0286	22.7179	0.4402	0.6491	1.5406
8	40	-40	0.0250	23.2051	0.4309	0.5801	1.7238

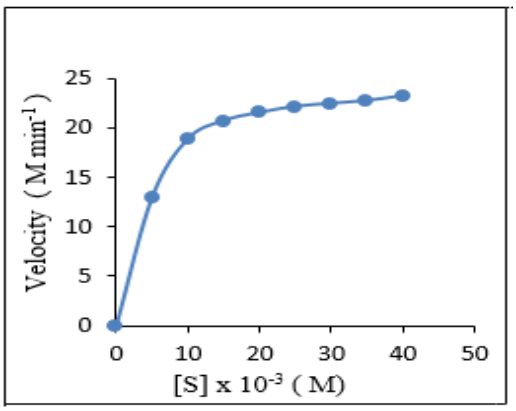

Figure 8 Michaelis-Menten plot used for graphic evaluation of V_{max} and K_m for crude peroxidase

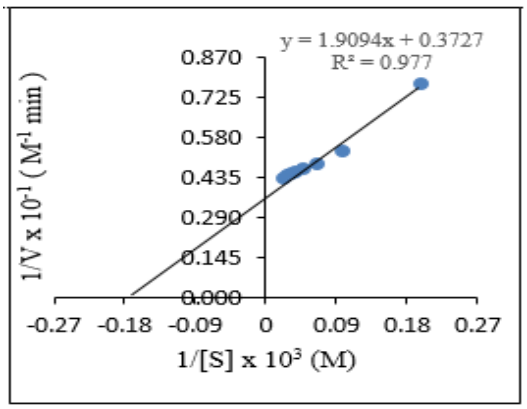

Figure 9 Lineweaver-Burk plot of 1/V *vs.* 1/[S] used for graphic evaluation V_{max} and K_m for crude peroxidase

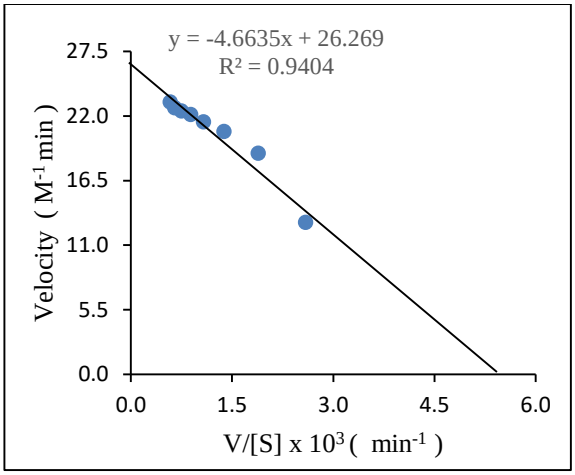

Figure 10 Eadie-Hofestee plot of V *vs.* V/[S] used for graphic evaluation of V_{max} and K_m for crude peroxidase

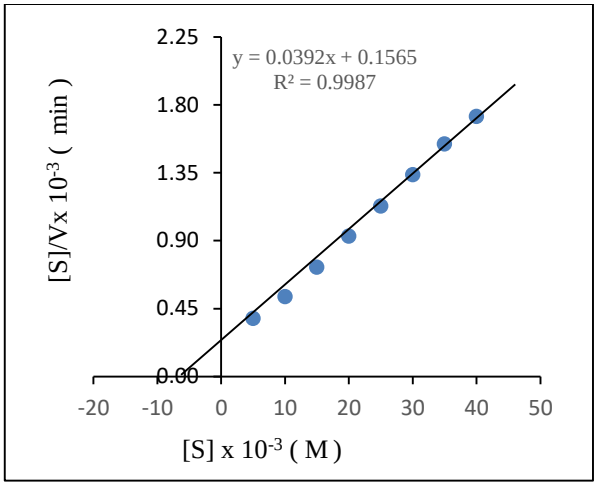

Figure 11 Hanes-Wilkinson plot of [S]/V *vs.* [S] used for graphic evaluation V_{max} and K_m for crude peroxidase

Table 9 Comparison of Kinetic Parameters of the Peroxidase Enzyme from Different Methods

No.	Methods	Graphical method		Linear regression method	
		V_{max} (M min^{-1})	$K_m \times 10^2$ (M)	V_{max} (M min^{-1})	$K_m \times 10^2$ (M)
1.	Michaelis-Menten	23.205	0.472	-	-
2.	Lineweaver-Burk	26.831	0.515	26.853	0.514
3.	Eadie-Hofstee	26.269	0.469	26.269	0.466
4.	Hanes-Wilkinson	26.837	0.421	25.510	0.397

Effect of Reaction Order on Peroxidase-catalyzed Reaction

The order of a chemical reaction with respect to the individual components is defined as power of the component concentration included into the rate equation. Depending on the substrates concentrations, the kinetic of an enzyme-catalyzed reaction may be described by the first-order rate equation (Bergmeyer, 1983).

The plot of Log $V/(V_{max}–V)$ *vs*. Log [S] will give a straight line from which reaction order (n) value can be computed from the slope (Table 10 and Figure 12). The reaction order (n) for peroxidase was calculated to be 1.22 proving that the reaction order was first order.

Table 10 Reaction Order for Peroxidase-catalyzed Reaction

No.	Log [S]	Log $V/(V_{max}-V)$
1	-2.3010	0.0462
2	-2.0000	0.5160
3	-1.8239	0.7250
4	-1.6989	0.8446
5	-1.6021	0.9537
6	-1.5229	1.0177
7	-1.4559	1.0841
7	-1.3979	1.2243

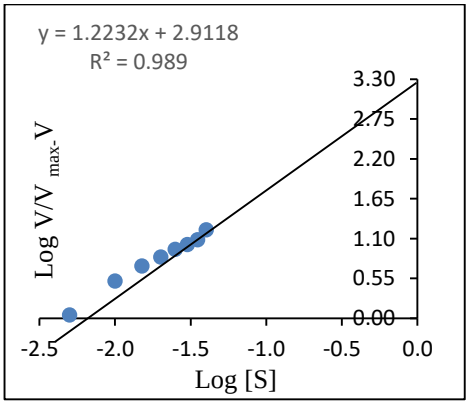

Figure 12 Plot of V/V_{max}-V as a function of log [S] of peroxidase-catalyzed reaction

Enzymatic Dye Decolourization

The decolourization of methyl orange (MO) by partially purified peroxidase from bitter gourd was studied by using spectrophotometric method. MO is a pH sensitive dye, red–orange under acidic conditions and yellow in neutral and alkaline conditions. Decolourization caused a change in colour from orange to yellow (Figure 13). Bitter gourd peroxidase was demonstrated a maximum dye decolourization efficiency of 80.42 % under the conditions of 4 h incubation at 30 °C using 2 mM of hydrogen peroxide, 0.2 mL crude bitter gourd peroxidase and 20 ppm methyl orange at pH 4.0.

The decolourization of MO may be thought to occur by the oxidation of the molecule by the partially purified peroxidase with H_2O_2. This oxidation is brought about by the enzyme by two sequential one-electron transfers (Veitch, 2004). This oxidation causes the formation of dye radicals and related chemical species that comprise the decolourized or bleached form of the dye (Coen *et al*., 2001). The susceptibility of the MO molecules to enzymatic oxidation would also depend on the pH of the reaction mixture.

Figure 13 (a) Methyl orange solution containing H_2O_2 (2 ppm) without enzyme (control) after 4 h
(b) Decolourized methyl orange solution containing H_2O_2 solution (2mM) using with peroxidase after 4 h

Conclusion

In this research, partially purified peroxidase enzyme was extracted from fresh bitter gourd by ammonium sulphate precipitation (20-70 %) method. The specific activity of peroxidase was 0.6361 U mg^{-1} and the enzyme was purified 1.99 fold over its crude extract. The optimum pH was 6.0 in phosphate buffer and optimum temperature of peroxidase was 40 ˚C. The activation energy for peroxidase-catalyzed reaction was calculated to be 3.592 kcal mol^{-1} between 10 and 40 ˚C and thus at lower temperatures, peroxidase enzyme will be relatively stable. K_m and V_{max} for guaiacol by Lineweaver-Burk was 0.515 x 10^{-2} M and 26.831 M min^{-1}, respectively, by graphical method and 0.514 x 10^{-2} M and 26.853 M min^{-1} by linear regression method. K_m and V_{max} values by other methods such as Eadie-Hofstee and Hanes-Wilkinson were also determined and found to agree with each other. The reaction order for peroxidase-catalyzed reaction of conversion of guaiacol to tetraguaiacol was found to be first order. Bitter gourd peroxidase was demonstrated a maximum dye decolourization efficiency of 80.42 % under the conditions of 4 h incubation at 30 °C using 2 mM of hydrogen peroxide, 0.2 mL crude bitter gourd peroxidase and 20 ppm methyl orange at pH 4.0.

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