

# Characterization of Partially Purified Peroxidase from Fingerroot (*Boesenbergia Rotunda* (L.) Mansf.)

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**Abstract**—Peroxidase from fingerroot was partially purified and characterized for potential use in analytical applications. The procedure began with crude extract preparation, followed by ammonium sulfate fractionation and concanavalin A-sepharose 4B affinity chromatography. The fraction of protein precipitated at 20-40% saturation of ammonium sulfate possessed the highest specific activity of 7.74 units/ mg. This fraction was subsequently purified further using affinity binding of peroxidase, a glycosylated enzyme, to Concanavalin A-sepharose 4B column. The chromatographic step produced peroxidase with specific activity of 55.33 units/ mg and resulted in 19.34 fold of purification. Investigation on optimal conditions revealed pH optimum to be at 6 and temperature optimum to be at 40 °C. After 5 hour incubation fingerroot peroxidase retained 60% of activity at pH 6 and 40 °C. Activity of the enzyme rapidly dropped at pH 2, while temperature at 70 °C and above inactivated the enzyme within the first hour. At concentration of 5 mM CaCl<sub>2</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>, NaCl and ZnCl<sub>2</sub> did not show notable effect on peroxidase activity, whereas CuCl<sub>2</sub> and FeCl<sub>2</sub> moderately inhibited the activity of peroxidase. AlCl<sub>3</sub> and FeCl<sub>3</sub> at 5 mM highly inhibited the activity of the enzyme up to 70%.

**Index Terms**—peroxidase, fingerroot, purification, characterization, specific activity

## I. INTRODUCTION

Peroxidases (EC 1.11.1.7, donor: hydrogen peroxide oxidoreductase) are heme containing enzymes that catalyze the oxidation of many organic and inorganic substrates at the expense of hydrogen peroxide. Peroxidases are widely distributed in nature. Plant peroxidases are involved mainly in cell elongation, lignification and defense mechanism. The most extensively studied peroxidase is from horseradish (*Armoracia rusticana*) [1]. Horseradish peroxidase has long been used for analytical applications in biosensors and diagnostic kits for quantitative analysis of cholesterol,

glucose and uric acid [2]. It is often used as a reporter enzyme in the enzyme-linked immunosorbent assay (ELISA) due to its ability to catalyze the color change of chromogenic substances upon the presence of hydrogen peroxide at low concentrations and its relatively good stability [3]. Horseradish Peroxidase can also be conjugated with avidin or streptavidin for using in the avidin (streptavidin)-biotin interaction as a detection system [4]. Additional applications of horseradish peroxidase include pollution monitoring, waste treatment [5], and aromatic compound synthesis [6]. Although, horseradish peroxidase dominates the field in analytical applications, enzymes of this type from other sources have received some attention. For example, soybean peroxidase has become commercially available and utilized. Berlina *et al.* developed an enzyme immunoassay kit for detection of sulfamethoxypyridazine using soybean peroxidase [7]. Agostini *et al.* purified peroxidases from roots and hairy root cultures of turnip for use in preparation of a uric acid diagnostic test kit [8]. Screening of peroxidase activity in topical plants is useful for finding alternative and/or economical sources of peroxidase for production of the enzyme. Our research group has been particularly interested in the screening of useful enzymes from fingerroot (*Boesenbergia rotunda* or formerly known as *Boesenbergia pandurata*), a Southeast Asian herb. Fresh fingerroot has unique aroma and slightly pungent taste. It is commonly used as a spice in condiments and as food ingredient [9]. Fingerroot is utilized as anti-dysentery and a carminative in traditional Thai medicine for the treatment of several diseases such as aphthous ulcer, dry mouth, stomach discomfort, leucorrhoea and dysentery [10]. Fingerroot also exhibits anti-bacterial [11], anti-fungal [12], anti-inflammatory [13], and anti-tumor [14] properties. Previous investigations have revealed that the rhizomes of fingerroot contain essential oils and chemical compounds including cineol, boesenbergin A, and camphor. Moreover, several studies have shown that some of the compounds, such as flavanoids and chalcones obtained

from this plant had appreciable inhibitory activity against HIV-1 protease [15] and dengue-2 virus NS3 protease [16]. Since these compounds may be produced as part of the plant defense mechanism, it is likely that this plant may produce peroxidase, one of enzymes involved in such mechanism. It was our interest to study peroxidase from this source. Peroxidase from fingerroot was partially purified and characterized. Its characteristics including pH-activity, pH-stability, thermal-activity, thermal-stability and effect of metal ions on activity are reported in this article.

## II. METHODOLOGY

### A. Partial Purification of Peroxidase from Fingerroot

- **Extraction:** Fingerroot, *Boesenbergia rotunda* (L.) Mansf., used in this study was purchased from a local supplier in Chiang Mai, Thailand. The fresh roots were cut into small pieces and blended with 10 mM phosphate buffer, pH 6 at 8:1 weight per volume ratio. The homogenates were filtered through cheesecloth and centrifuged at 6000 xg for 20 min at 4 °C to remove insoluble debris. This crude extract was collected and analyzed for peroxidase activity and protein content.
- **Protein Precipitation by Ammonium Sulfate:** Protein precipitation of the fingerroot extract was carried out stepwise using ammonium sulfate to obtain saturation (Saturation) at 20%, 40%, 60%, 80% and 90%. After addition of ammonium sulfate in each step the sample was centrifuged at 6000 xg for 20 min. The precipitate was collected and the supernatant was used for the next precipitation step. Preliminary screening of peroxidase activity was performed for the crude extract and different dialyzed fractions (0-20% Saturation, 20-40% Saturation, 40-60% Saturation, 60-80% Saturation, 80-90% Saturation and supernatant). The dialysate with the highest peroxidase specific activity was collected and stored at 4 °C for further purification.
- **Concanavalin A-sepharose 4B affinity chromatography:** The Concanavalin A-sepharose 4B column (1.4 x 18.0 cm) was equilibrated with 0.01 M phosphate buffer, pH 6 containing 0.5 mM each of MnCl<sub>2</sub>, MgCl<sub>2</sub>, CaCl<sub>2</sub> and NaCl. The dialysate with the highest specific activity of peroxidase (20-40% Saturation fraction) was applied onto the column. The eluent buffer was a gradient of 0.0-0.5 mM D-(+)-mannose in similar buffer. The flow rate was 2.5 ml/ min and each fraction was collected for 2.0 ml. The fractions with the highest specific activity from each run were pooled together for further analysis.

### B. Peroxidase Activity Assay

All enzyme samples were assayed following the method of Agostini *et al.* [8] with slight modification. Peroxidase activity was determined using guaiacol as substrate. The reaction mixture contained guaiacol (15

mM, 1 ml), H<sub>2</sub>O<sub>2</sub> (3 mM, 1 ml), phosphate buffer (10 mM, pH 6, 950 µl) and enzyme samples (50 µl) in a total volume of 3 ml. The reaction was monitored at 25 °C by continuously measuring the absorbance at 470 nm for 1 min. All reactions were repeated in triplicate. One unit of peroxidase activity was defined as the amount of enzyme catalyzing the oxidation of 1 µM of guaiacol per min at 25 °C, pH 6. The activity was calculated using the equation (1) where  $\Delta A/t$  is the variation of absorbance at 470 nm per min,  $V_t$  is the total reaction volume (ml),  $D_f$  is the dilution factor,  $\epsilon$  is the guaiacol molar extinction coefficient; 26.6 mM<sup>-1</sup> cm<sup>-1</sup>,  $p$  is the cuvette path length (1 cm),  $S_f$  is the stoichiometric factor; 0.25 and  $S_v$  is the sample volume (µl).

$$\text{Peroxidase activity (unit/ml)} = \frac{\Delta A \times V_t \times D_f \times 1000}{t \times \epsilon \times p \times S_f \times S_v} \quad (1)$$

### C. Protein Content Determination

Protein content was determined according to Bradford method [17], using bovine serum albumin as a protein standard.

### D. Statistical Analysis

The data was analyzed statistically using Microsoft Excel 2000.

### E. pH-activity Profile

Determination of the pH activity of partially purified peroxidase was performed at 25 °C using 10 mM buffer at various pHs ranging from 2 to 10. Sodium citrate buffer was used for pH 2-5. Sodium phosphate buffer was used for pH 6-7 and sodium borate buffer was used for pH 8-10.

### F. Thermal-activity Profile

Determination of temperature optimum of partially purified peroxidase was carried out by incubating enzyme solution and substrate individually at the same constant temperature of 30, 40, 50, 60, 70, 80 and 90 °C in 10 mM phosphate buffer, pH 6 for 10 min prior to mixing and activity monitoring at standard condition.

### G. pH-stability Profile

pH stability of partially purified peroxidase was analyzed by incubating the enzyme solution in the appropriate buffer at pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 at 25 °C in the range of 1-5 h. The incubated enzymes were then assayed for peroxidase activity in 10 mM phosphate buffer at pH 6 following standard protocol.

### H. Thermal-stability Profile

Samples of partially purified peroxidase in 10 mM phosphate buffer, pH 6.0 were maintained at different temperatures (30, 40, 50, 60, 70, 80 and 90 °C) in the range of 1-5 h. The activity of the incubated enzyme was then determined according to standard protocol at 25 °C.

### I. Effect of Metal Ions on Activity

Selected metal ions were tested for effect on the activity of peroxidase by pre-incubating the partially purified peroxidase with individual ions (AlCl<sub>3</sub>, CaCl<sub>2</sub>,

CuCl<sub>2</sub>, FeCl<sub>2</sub>, FeCl<sub>3</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>, NaCl and ZnCl<sub>2</sub>) in 10 mM phosphate buffer, pH 6 for 10 min [18]. The activity of the enzyme was then determined by adding appropriate volume of pre-incubated enzyme into substrate solution under standard assay conditions. The peroxidase activity of the pre-incubated enzyme in the presence of each metal ion was compared with that in the absence (control) of metal ions, which was defined as the 100% level.

#### J. Native Polyacrylamide Gel Electrophoresis

In gel detection of peroxidase was performed using polyacrylamide gel (4.0% for stacking gel and 7.5% for resolving gel). Electrophoresis was carried out using a mini-VE vertical slab-gel unit (Amersham Bioscience, USA) with constant current of 40 mA. The native gel was incubated with 160 mM guaiacol and 160 mM hydrogen peroxide in 10 mM phosphate buffer, pH 6.0 at 25 °C until the orange band of tetraguaiacol appeared locating protein with peroxidase-like activity (30 min). This protocol was modified from that described by Johri et al. [19]. The gel was rinsed with distilled water for 30 min and photographed, followed by placing the gel in the protein staining solution (0.025% Coomassie Brilliant Blue R-250, 40% methanol and 7% acetic acid) overnight. Destaining protocol was carried out until the background of the gel was clear and ready to be photographed.

### III. RESULTS AND DISCUSSION

#### A. Partial Purification of Fingerroot Peroxidase

Partial purification of peroxidase from fingerroot began with preparation of the crude extract. Ammonium sulfate was subsequently applied to the extract stepwise to obtain protein precipitate at different ranges of saturation. It was expected that each different fraction would result in different values of specific activity due to variation in the enzymatic activity and protein content. Peroxidase activity was determined using guaiacol as a substrate in the presence of hydrogen peroxide at final

reaction concentration of 5 mM and 1 mM, respectively. Peroxidase catalyzes the oxidation of the substrate from colorless to orange complex product, tetraguaiacol. Four moles of guaiacol produce one mole of the tetraguaiacol. This reaction was measured with a spectrophotometer at 470 nm at 25 °C. This reaction was carried out for 3 min. The rate of reaction ( $\Delta 470/\text{min}$ ) was calculated and reported as peroxidase activity using molar extinction coefficient as described for equation (1). One unit of peroxidase activity is defined as the amount of enzyme catalyzing the oxidation of 1  $\mu\text{mole}$  of guaiacol in 1 min. The protein content was determined following the method of Bradford [17]. Peroxidase was extracted from fingerroot using 10 mM phosphate buffer, pH 6. Data showed that the crude extract had total protein content of 577.86 mg and total peroxidase activity of 1653.54 units resulting in specific activity of 2.86 units/mg as shown in Table I. Ammonium sulfate fractionation results revealed that peroxidase did not precipitate out as much in the first fraction (0-20% Sat, specific activity of 1.87 units/mg) as in the second fraction (20-40% Saturation) where peroxidase was present with the highest specific activity of 7.74 units/mg. The fraction with the second highest specific activity was the fraction of 40-60% Saturation. This fraction also contained proteins with peroxidase-like activity. The 40-60% Saturation fraction had specific activity of 6.21 units/mg. When percent saturation of ammonium sulfate was increased to 80%, little peroxidase activity was found resulting in a specific activity of 1.43 units/mg. Interestingly when the saturation was increased to 90% there was another jump in peroxidase activity to 3.57 units/mg suggesting that there might be more than one type of peroxidase in the extract. This observation was later supported by the in-gel detection of peroxidase revealing different fingerroot peroxidase isoenzymes (see Fig. 1). Lastly with little protein left in the solution, the supernatant with the remaining peroxidase possessed specific activity of 2.00 units/mg.

TABLE I. AMMONIUM SULFATE FRACTIONATION OF PEROXIDASE FROM FINGERROOT

Sample	Total activity (units)	Activity recovery (%)	Total protein(mg)	Specific activity(units/ mg)	Purification fold
Crude	1653.54	100	577.86	2.86	1
0-20%	25.45	1.54	13.58	1.87	0.66
20-40%	493.96	29.87	63.81	7.74	2.71
40-60%	283.48	17.14	45.62	6.21	2.17
60-80%	13.91	0.84	9.73	1.43	0.50
80-90%	1.25	0.08	0.35	3.57	1.25
Super	0.02	0.001	0.01	2.00	0.70

Values represent the mean of three replicates. Percentage ranges indicate the percentage of saturation of ammonium sulfate used in each step.

Native-PAGE was selected as a mean for enzyme detection and for confirmation of the results from precipitation of peroxidase using different percentages of ammonium sulfate saturation. The non-denaturing gel stained by guaiacol (Fig. 1) showed that lane 5 which contained proteins precipitated out in the 20-40% Saturation fraction appeared with the most intense orange bands of tetraguaiacol, followed by lane 6 which contained proteins precipitated out in the 40-60%

Saturation fraction. Results from this gel agreed well with the findings reported in Table I.

It is notable that when the same gel was stained using Coomassie Brilliant Blue, a protein dye, as shown in fig. 2, lane 1, BSA used as a negative control was not stained. This validates that the in-gel detection using guaiacol in the presence of hydrogen peroxide is specific to proteins with peroxidase activity. HRP in lane 2 was used as a positive control and showed up well with an intense

orange band. The migration distance of HRP and peroxidase from fingerroot peroxidase were different. Fingerroot peroxidase has higher relative mobility than that of horseradish peroxidase signifying that it either had lower molecular weight or had more negative charge on the surface. Since proteins in non-denaturing gel retain their native structures, this speculation will need to be proved by other means. When percentage of ammonium sulfate reach 80%, different protein bands with peroxidase activity appeared indicating another group of peroxidase with either lower molecular weight or more negative charge. While the addition of molecular weight may come from amino acid sequence difference, it may also come from the change in glycosylation. The extra negatively charged amino acid residues may also attribute to the relative mobility and the high solubility in salt solution requiring high percentages of ammonium sulfate saturation to precipitate them out. When the isoenzymes present in the fingerroot extract can be well separated, their structures and characteristics will be addressed better.

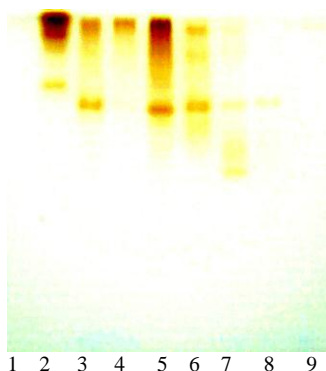


Figure 1. Native-PAGE of protein samples resulting from ammonium sulfate fractionation stained with guaiacol in the presence of H<sub>2</sub>O<sub>2</sub> for activity indication. Polyacrylamide resolving gel of 7.5% was used and the staining method was modified from that described by Johri *et al.* [19]. The samples include bovine serum albumin, 0.25 µg (lane 1); horseradish peroxidase, 0.31 µg (lane 2); crude extract, 15 µg (lane 3); 0-20% Saturation, 15 µg (lane 4); 20-40% Saturation, 15 µg (lane 5); 40-60% Saturation, 15 µg (lane 6); 60-80% Saturation, 15 µg (lane 7); 80-90% Saturation, 1.2 µg (lane 8) and Supernatant, 0.025 µg (lane 9).

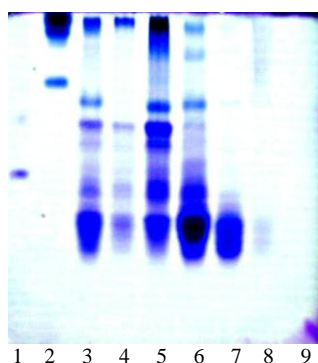


Figure 2. Native-PAGE of protein samples resulted from ammonium sulfate fractionation stained with Coomassie Brilliant Blue R-250. After activity detection, the same gel (fig. 1) was subsequently stained for visualization of protein bands in samples. The samples include bovine serum albumin, 0.25 µg (lane 1); horseradish peroxidase, 0.31 µg (lane 2); crude extract, 15 µg (lane 3); 0-20% Saturation, 15 µg (lane 4); 20-40% Saturation, 15 µg (lane 5); 40-60% Saturation, 15 µg (lane 6); 60-80% Saturation, 15 µg (lane 7); 80-90% Saturation, 1.2 µg (lane 8) and Supernatant, 0.025 µg (lane 9).

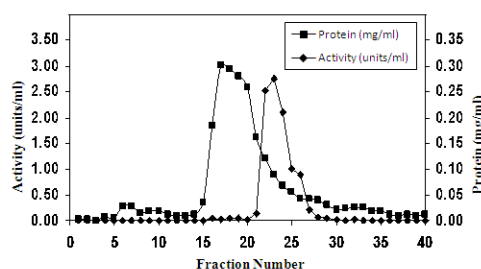


Figure 3. Representative of elution profile of Concanavalin A-sepharose 4B affinity chromatography of 20-40% Saturation protein sample. The fractions with the highest specific activity from each run were pooled together for further analysis.

According to specific activity and recovery yield, the protein sample obtained from precipitation using ammonium sulfate at 20-40% Saturation was selected for further purification using Concanavalin A-sepharose 4B affinity chromatography. This method was selected in an attempt to use the immobilized Concanavalin A, a lectin from jack fruit seed with binding specificity to glucose and mannose, to purify peroxidase which is a glycoprotein following the method described by López-Arnaldos *et al.* [20]. The protein sample was loaded onto Concanavalin A-Sepharose 4B column (1.4 x 18 cm) and a solution of 0-0.5 mM D-(+)-mannose in 10 mM phosphate buffer, pH 6 containing 5.0 mM of each MnCl<sub>2</sub>, MgCl<sub>2</sub> and CaCl<sub>2</sub> was used to elute out the proteins from the column with flow rate of 0.5 ml/ min and the sample was collected 2.0 ml per fraction. Each fraction was assayed for peroxidase activity and protein content. Representative elution profile of peroxidase separation by Concanavalin A-Sepharose 4B chromatography is illustrated in Fig. 3. The elution began with the buffer in the absence of D-(+)-mannose up to fraction number 10. For every next 10 fraction the buffer was changed to 1 mM of D-(+)-mannose higher. When the elution reached fraction number 60, another 10 fractions were collected using buffer containing 5 mM of D-(+)-mannose. After fraction number 40, there was no detectable activity of peroxidase. The representative profile shows the range where peroxidase activity was detected.

After Concanavalin A-sepharose 4B affinity chromatography, the specific activity of peroxidase was raised to 55.33 units/ mg resulting in approximately 19.34 fold of purification as shown in Table II. Although this method seemed to yield enough peroxidase for further characterization, additional chromatographic method is required for separation of isoenzymes for amino acid sequencing and post-translational modification analysis.

TABLE II. PARTIAL PURIFICATION OF FINGERROOT PEROXIADSE

Sample	Total Activity (units)	Activity recovery (%)	Total protein (mg)	Specific activity (units/mg)	Purification fold
Crude	1653.54	100	577.86	2.86	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	493.96	29.87	63.81	7.74	2.71
Affinity	14.94	0.90	0.27	55.33	19.34

Value shown for (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitate was obtained from 20-40% Saturation sample which was further purified using Concanavalin A-sepharose 4B affinity chromatography.

Native-PAGE shown in Fig. 4A confirmed the presence of peroxidase in all the purification steps where the gel was stained with guaiacol in the presence of hydrogen peroxide. The orange bands indicated the presence of peroxidase in those fractions. Subsequently the same gel was stained using Coomassie Brilliant Blue R-250 to observe all detectable proteins. Fig. 4B reveals the decrease of protein population resulting from the partial purification. It is possible that some of the proteins that eluted out in the same fraction as peroxidase were glycosylated and also bound to the column. Hence, another type of chromatography such as size exclusion chromatography would help increase specific activity when applied prior to the affinity chromatography.

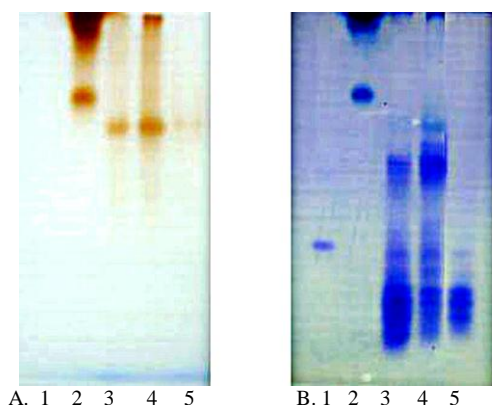


Figure 4. Native-PAGE of protein samples resulting from each purification step stained with guaiacol in the presence of  $H_2O_2$  for activity indication (A) and with Coomassie Brilliant Blue R-250 (B). After activity detection, the same non-denaturing gel was subsequently stained for visualization of protein bands in the samples. The samples include bovine serum albumin, 0.25  $\mu$ g (lane 1); horseradish peroxidase, 0.31  $\mu$ g (lane 2); crude extract, 15  $\mu$ g (lane 3); 20-40% Saturation, 15  $\mu$ g (lane 4) and pooled samples from Concanavalin A-sepharose 4B affinity chromatography, 0.48  $\mu$ g (lane 5)

## B. Characterization of Partially Purified Fingerroot Peroxidase

### 1) pH-activity profile

The fractions with the highest specific activity from each run of Concanavalin A-sepharose 4B affinity chromatography were pooled together for characterization including pH-activity profile, thermal-activity profile, pH-stability profile, thermal stability profile and effect of metal ions on activity investigation. Fig. 5 shows relative activity of fingerroot peroxidase at different pHs. Experimental data revealed that peroxidase was able to function in pH range of 3-10. However, the enzyme was mostly active at pH 6. Activity of enzyme assessed at pH 3 and 10 was only about 50% and 46%, respectively, while at pH 2 fingerroot peroxidase became inactive. Fingerroot peroxidase had maximum activity at pH 6 suggesting that the enzyme can function in slightly acidic environment. Peroxidases purified from different sources have their optimum pH mostly in the region of 5-6. The optimum pH for spring cabbage was at pH 6 [21], the same as that found in bitter gourd [22], while the optimum pH for peroxidase from Chinese cabbage roots was at pH 5 [23] and pH 5.5 for *Metroxylon sagu* [18]. When the pH was lower than 4 or over 7, fingerroot

peroxidase activity dropped. At extreme pH conditions, the active site of the enzyme can be irreversibly denatured resulting in the loss of catalytic ability.

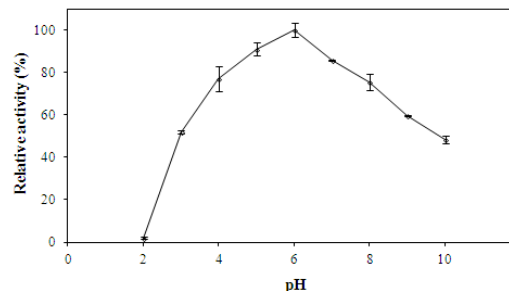


Figure 5. pH-activity profile of partially purified fingerroot peroxidase. The optimum pH was determined at 25°C using 10 mM buffer at various pHs ranging from 2 to 10. Sodium citrate buffer was used for pH 2-5. Sodium phosphate buffer was used for pH 6-7 and sodium borate buffer was used for pH 8-10. Peroxidase was that obtained from pooled samples from Concanavalin A-sepharose 4B affinity chromatography.

### 2) Thermal-activity profile

Optimal temperature of the partially purified peroxidase was examined at different temperatures varying from 30 to 90 °C. Enzyme and substrate were incubated separately in the same waterbath for 10 min at constant temperature before assaying under standard condition. The results show that the partially purified fingerroot peroxidase was active in temperature range of 30 to 70 °C, while a gradual decrease in the activity was shown at temperatures above 70 °C. Peroxidase activity was optimal at 40 °C as shown in Fig. 6.

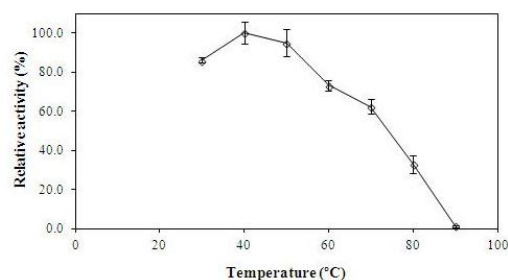


Figure 6. Thermal-activity profile of partially purified fingerroot peroxidase. Peroxidase obtained from pooled sample from Concanavalin A-sepharose 4B affinity chromatography and substrate were individually incubated at the same constant temperatures of 30, 40, 50, 60, 70, 80 and 90 °C in 10 mM phosphate buffer, pH 6.0 for 10 min prior to mixing and activity monitoring at standard condition.

The optimal temperature of fingerroot peroxidase at 40 °C is similar to that of spring cabbage [21] and bitter gourd [22]. At 70 °C and above, fingerroot peroxidase rapidly lost its activity possibly due to thermal denaturation.

### 3) pH-stability profile

pH stability of partially purified peroxidase was analyzed by incubating the enzyme at different pHs at room temperature for up to 5 h. The pre-incubated enzyme solutions were then assayed for peroxidase activity every hour. The results showed that the enzyme was stable in the range of pH 4-10 for 5 h where the relative activity was at 50% at the fifth hour. At pH 3, peroxidase activity decreased from 100% to 45% at 3 h,

and continued to maintain at 37% at 5 h. Moreover, enzymatic activity decreased rapidly at pH 2.0 within 1 h and remained at 2.6% from 3 to 5 h as shown in Fig. 7. Our finding is common with studies reported on *Withania somnifera* (AGB 002) peroxidase with stability in the pH range of 3-9 [19], while oil palm leaf peroxidase was reported to be stable in pH range of 5-10 [24].

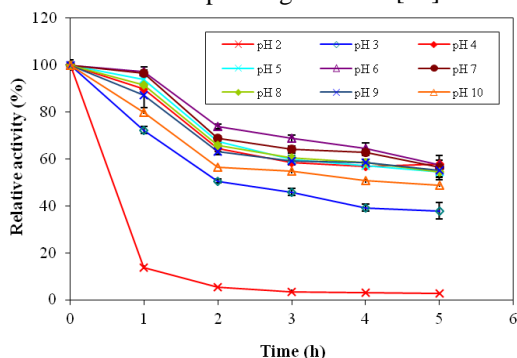


Figure 7. pH-stability profile of partially purified fingerroot peroxidase.

Peroxidase obtained from pooled sample from Concanavalin A-sepharose 4B affinity chromatography was incubated in the appropriate buffer at pHs 2, 3, 4, 5, 6, 7, 8, 9 and 10 at 25 °C in the range of 1-5 h. The incubated enzymes were then assayed for peroxidase activity in 10 mM phosphate buffer, pH 6 following standard protocol at each time interval.

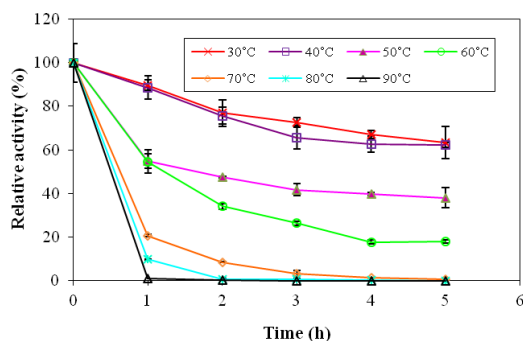


Figure 8. Thermal-stability profile of partially purified fingerroot peroxidase. Peroxidase obtained from pooled samples from Concanavalin A-sepharose 4B affinity chromatography in 10 mM phosphate buffer, pH 6.0 were maintained at different temperatures (30, 40, 50, 60, 70, 80 and 90 °C) in the range of 1-5 h. The activity of the incubated enzyme was then determined at each time interval according to standard protocol at 25 °C.

#### 4) Thermal-stability profile

In the study of temperature stability, partially purified peroxidase in 10 mM phosphate buffer, pH 6 was maintained at different temperatures up to 5 h (see Fig. 8). The incubated enzymes were then assayed for peroxidase activity every hour. It was demonstrated that, peroxidase maintained its activity in the temperature range of 30 to 40 °C; where at 5 h the relative activity was at 60%. Activity of peroxidase pre-incubated at 50.0 °C and 60 °C decreased from 100% to 47% and 34% at 3 h, but continued to maintain at above 37% and 18% at 5 h, respectively. Moreover, enzymatic activity decreased rapidly for those pre-incubated at 70, 80 and 90 °C within the first hour and approached inactivity after 2 h. Our results showed that fingerroot peroxidase was stable at temperatures between 30 to 40 °C for 5 h and at 50 to 60 °C for 1 h which similar to peroxidase from leaves of

*Ipomoea palmetta* [25]. When peroxidase maintains above 50% of its activity, the enzyme is considered stable at that temperature [26].

The variability in the temperature stability of peroxidase can be attributed mainly to the particular intra-molecular forces involved in the structure and post-translational modifications.

#### 5) Effect of metal ions on peroxidase activity

The effects of some metal ions on activity of partially purified peroxidase, were determined by pre-incubating the partially purified peroxidase with individual ions ( $\text{AlCl}_3$ ,  $\text{CaCl}_2$ ,  $\text{CuCl}_2$ ,  $\text{FeCl}_2$ ,  $\text{FeCl}_3$ ,  $\text{MgCl}_2$ ,  $\text{MnCl}_2$ ,  $\text{NaCl}$  and  $\text{ZnCl}_2$ ) in 10 mM phosphate buffer, pH 6 for 10 min. Relative activity could be compared with those without metal ions (control). Table III shows relative activity of fingerroot peroxidase after incubation with various ions. At concentration of 5 mM  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{MnCl}_2$ ,  $\text{NaCl}$  and  $\text{ZnCl}_2$  did not show notable effect on peroxidase activity. Whereas, at 5 mM  $\text{CuCl}_2$  30% of peroxidase activity was lost. At concentration of 5 mM  $\text{AlCl}_3$ ,  $\text{FeCl}_3$  and  $\text{FeCl}_2$  highly inhibited the activity of enzyme giving approximately 39%, 33% and 52% of the activity compared to the control, respectively.

TABLE III. EFFECT OF METAL IONS ON PEROXIDASE ACTIVITY

Metal ions (5 mM)	Relative activity (%)
$\text{AlCl}_3$	38.6 ± 0.05
$\text{CaCl}_2$	96.4 ± 0.04
$\text{CuCl}_2$	70.4 ± 0.25
$\text{FeCl}_2$	52.3 ± 0.19
$\text{FeCl}_3$	33.4 ± 0.11
$\text{MgCl}_2$	90.5 ± 0.23
$\text{MnCl}_2$	95.0 ± 0.60
$\text{NaCl}$	99.9 ± 0.07
$\text{ZnCl}_2$	94.0 ± 0.48

Values represent the mean of three replicates. Peroxidase obtained from pooled sample from Concanavalin A-sepharose 4B affinity chromatography was pre-incubated with individual ions in 10 mM phosphate buffer, pH 6 for 10 min prior to activity determination.

Inhibition of fingerroot peroxidase by these metal ions is likely to be the non-competitive type. Similar effects have been observed in peroxidases from other sources. The inhibitory effect of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  ions was also observed for *Ipomoea cairica* (L) [27], while Splittgerber and Tappel reported that  $\text{Al}^{3+}$  decreased peroxidase activity [28]. Our preliminary findings revealed that fingerroot peroxidase catalyzes the reaction best at pH 6 and 40 °C when guaiacol was used as substrate. The enzyme was stable at pH 3-10 and 30-50 °C. The enzyme was only affected greatly by few metal ions such as  $\text{Al}^{3+}$ ,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ . These characteristics are relatively similar to those of horseradish peroxidase [1]-[3] suggesting that fingerroot is another alternative source of peroxidase that may be employed in analytical applications.

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