

Full Length Research Paper

# Purification and partial characterization of peroxidase from lettuce stems

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Accepted 23 January, 2012

**Peroxidase (EC1.11.1.7) was purified to homogeneity from lettuce (*Lactuca sativa* L.) stems by means of 40 to 80% ammonium sulfate precipitation, Sephadex G-100 gel filtration and affinity chromatography with concanavalin A. Peroxidase was purified 17.92-fold with 2.67% recovery and its molecular mass was 35 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Its optimum temperature and pH were 45°C and 5.0. In addition, it showed moderate thermostability at 60°C. Its  $K_m$  for guaiacol, pyrogallol, 2, 2'-azino-bis-(3-ethylbenzthiazolin-6-sulfonate) and catechol was 4.74, 1.96, 3.75 and 2.95 mM, respectively. Its activity was inhibited by metal ions ( $Fe^{3+}$ ,  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$  and  $Mn^{2+}$ ) and organic solvents (methyl alcohol, ethanol and acetone).**

**Key words:** Lettuce, partial characterization, peroxidase, protein purification, thermostability.

## INTRODUCTION

Peroxidases (EC1.11.1.7) are heme-containing enzymes that oxidize a wide variety of organic and inorganic substrates by reducing  $H_2O_2$  and peroxides (Kvaratskhelia et al., 1997). Peroxidases, ubiquitously existing in nature, are divided into three groups according to their amino acid sequences: class I (ascorbate type), class II (fungal secretory) and class III (guaiacol type, plant secretory) (Das et al., 2011). Among them, most of the plants secretory peroxidases are glycosylated proteins (Kvaratskhelia et al., 1997; Johansson et al., 1992). They play critical roles in physiological functions such as lignification, cell wall metabolism, enhancing plant resistance, eliminating  $H_2O_2$  injures and participating auxin catabolism (Wojtaszek, 1997; Boka and Orban, 2007; Hiraga et al., 2001; Lagrimini, 1997). Products from these enzymes are also commonly used in

pharmaceutical industry, waste water treatment, bio-sensor construction and food industry (Jia et al., 2002; Köksal and Gülçin, 2008; Lavery et al., 2010).

On the other hand, peroxidases are considered as a factor responsible for the deterioration when vegetables or fruits are processed such as shredding and cutting. Peroxidases affect their organoleptic properties such as color, taste and aroma, causing off-flavors, off-colors, browning and nutritional damage (Mdluli, 2005). Lettuce, as a common vegetable, is often used for salad or Chinese cold dish. Since peroxidase influences its quality and shelf-life, it is important to explore lettuce peroxidase characterization.

It was reported that plant secretory peroxidases had been purified and characterized from lots of plants such as horseradish, soybean, bitter melon and tea (Kvaratskhelia et al., 1997; Lavery et al., 2010; Fatima and Husain, 2008; Silva and Franco, 2000). Among them, horseradish peroxidase was most widely investigated and used for commercial applications as tool enzyme in chemical analysis and enzyme-linked immunosorbent assay (ELISA) (Dunford, 1991). Some researchers had partially characterized lettuce peroxidase (Bestwick et al., 1998; Tomás-Barberán et al., 1997), while partial purifica-

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**Abbreviations:** ABTS, 2, 2'-azino-bis-(3-ethylbenzthiazolin-6-sulfonate); EC, enzyme code; U, Unit; RZ, Reinzhal value;  $K_{cat}$ , catalytic rate constant.

tion of this enzyme was also reported (Altunkaya and Gökmen, 2011). However, lettuce peroxidase has not been purified to homogeneity and most of its properties were not well characterized. Therefore, considering the important role of peroxidase in lettuce processing, the objectives of this study were to purify lettuce peroxidase and further investigate its properties.

## MATERIALS AND METHODS

Lettuce (*Lactuca sativa* L.) was obtained from a local market. Sephadex G-100 and concanavalin A were purchased from Amersham Biosciences Corporation.

### Enzyme isolation

One kilogram of fresh lettuce stems was homogenized at 0 to 4°C with 800 ml of 0.1 M Tris-HCl buffer (pH 7.4). After centrifuged at 10,000 × g for 10 min, the supernatant was pooled and added with solid ammonium sulfate to 40% saturation and was left overnight at 4°C. Then centrifuged again as stated earlier, the supernatant was pooled and added with solid ammonium sulfate to 80% saturation. The solution was kept overnight at 4°C again and centrifuged as stated earlier. The precipitate was dissolved in distilled water and dialyzed against the same buffer to remove ammonium sulfate.

The previous solution was subjected to gel filtration on Sephadex G-100 column (5×70 cm) previously equilibrated with 0.1 M Tris-HCl buffer (pH 7.4). The column was eluted in the same buffer at the flow rate of 1 ml/min. The eluted protein fractions were collected and peroxidase activity was measured. The fractions exhibiting high peroxidase activity were pooled. This procedure was repeated until the total of the solution was put through Sephadex G-100 column. All the fractions with high enzyme activity were pooled together and subjected to concanavalin A column (0.5×10 cm), which were equilibrated with 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub> and 0.5 mM NaCl. Elution was done with the buffer containing 0.15 mM each of glucose and mannose. The proteins were determined by the assay of Bradford (1976), and bovine serum albumin was used as standard.

### Peroxidase activity and substrate specificity assay

Assay of peroxidase activity and substrate specificity were performed according to the method by Köksal and Gülçin (2008). Briefly, to determine the peroxidase activity, the enzyme solution (50 µl) was added to a mixture of 1 ml 260 mM H<sub>2</sub>O<sub>2</sub> and 50 µl 20 mM guaiacol, and the mixture was adjusted to 2 ml by adding 0.1 M sodium phosphate buffer (pH 6.0). One unit of peroxidase activity was defined as 0.01 ΔA<sub>470</sub> per min (Fujita et al., 1997). To determine the substrate specificity, various substrates were used such as guaiacol, pyrogallol, 2, 2'-azino-bis-(3-ethylbenzthiazolin-6-sulfonate) (ABTS) and catechol while H<sub>2</sub>O<sub>2</sub> concentration was constant. The wavelengths used in this study were: 470 nm for guaiacol, 415 nm for pyrogallol and ABTS and 290 nm for catechol. The changes in absorbance were read using a UV-VIS spectrophotometer UVmini-1240 (Shimadzu Corp, Japan). *K<sub>m</sub>* and *V<sub>max</sub>* values determined with Lineweaver-Burk plot.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the Laemmli's system

(Laemmli, 1970), using 12.5% polyacryl amide gel.

### Effect of pH values

The optimum pH value for peroxidase was determined by assaying enzyme activity at different pH levels. The assay was performed by taking buffers at different pH such as 0.1 M sodium acetate buffer (pH 3.0 to 5.0) and 0.1 M sodium phosphate buffer (pH 5.5 to 6.5). Peroxidase activity was assayed by using guaiacol substrate as stated earlier.

### Effect of temperature

The optimum temperature of peroxidase was determined by assaying the enzyme with the difference that the activity was determined at different temperature from 20 to 60°C. The thermostability of peroxidase was measured by incubating the enzyme at 60°C and the activity was assayed every 10 min. Peroxidase activity was assayed by using guaiacol substrate as stated earlier.

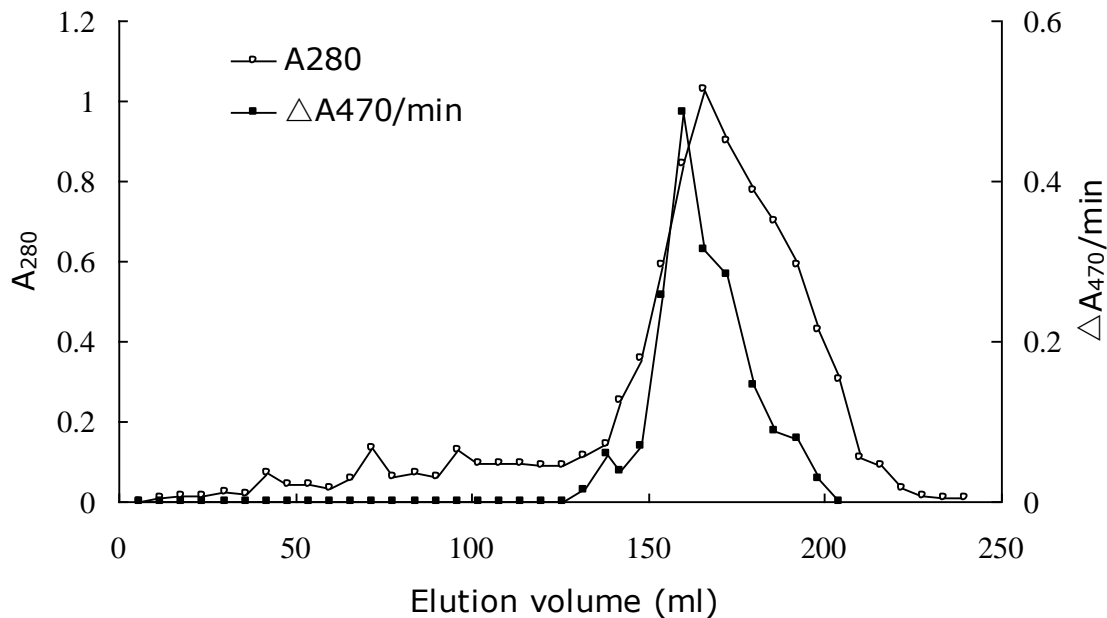
### Effect of organic solvents and metals

Peroxidase activity was determined in the presence of propylene glycol, methyl alcohol, ethanol and acetone at variable concentrations (2 to 25%) and Fe<sup>3+</sup>, Zn<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup> and Mo<sup>6+</sup> at 1 and 5 mM by using guaiacol substrate as stated earlier.

## RESULTS AND DISCUSSION

### Purification of lettuce peroxidase

After gel filtration on Sephadex G-100 column, four peaks of proteins, three small peaks followed by a large peak, were eluted from the column (Figure 1). The enzyme activity was detected in the last peak, indicating parts of proteins were removed by the gel filtration. This procedure allowed peroxidase to be purified 2.71-fold, compared to the crude extract (Table 1). The fractions were loaded further onto concanavalin A column, and peroxidase was eluted with 0.1 M Tris-HCl buffer (pH 7.4) containing 0.15 M glucose and mannose. The fractions with high activity were pooled (Figure 2). Finally, the enzyme was purified to 17.92-fold with recovery of 2.67%. The specific activity of the purified enzyme was 51,136.10 U/mg with RZ value 3.18 (Table 1). To determine the enzyme purity, four micro grams of the enzyme solution protein were used for SDS-PAGE. It showed one single band with molecular mass 35 kDa (Figure 3). The molecular mass for most peroxidases was between 32 and 45 kDa (Rompel et al., 2007), such as 40 kDa in horseradish root (Lavery et al., 2010), 44 kDa in auliflower buds (Köksal and Gülçin, 2008), 39 kDa in chick pea (Bhatti et al., 2006) and 36.5 kDa in garlic (Marzouki et al., 2005). It was indicated that the peroxidase from lettuce stems was in the regular range of molecular masses between 32 and 45 kDa.



**Figure 1.** Chromatography of lettuce peroxidase on Sephadex G-100 Column. The crude extract after ammonium sulphate precipitation were dialyzed and passed through Sephadex G-100 column. The column was equilibrated with 0.1 M Tris-HCl buffer (pH 7.4).

**Table 1.** Purification of peroxidase from lettuce stems.

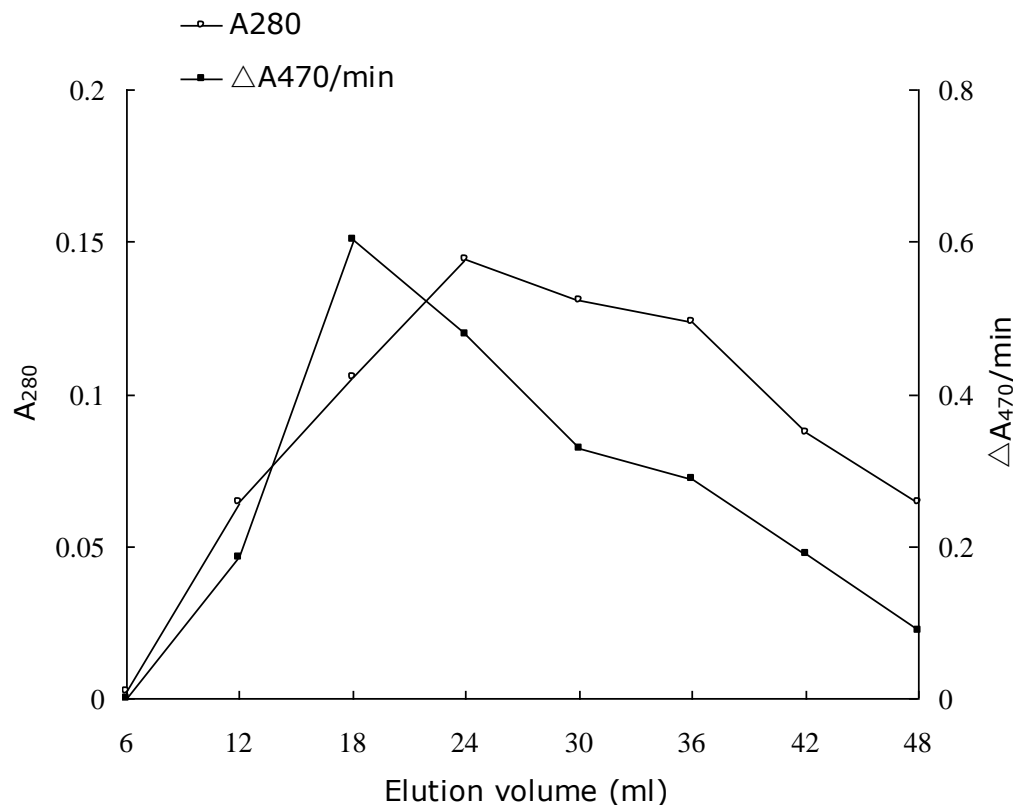
Step	Total volume (ml)	Total activity (U/min)	Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)	RZ value
Crude extract	1,700	2,556,800	895.81	2,854.17	100	1	0.12
40 - 80% ammonium sulfate	60	1,934,400	512.84	3,771.93	75.66	1.32	0.23
G-100 filtration	185	842,120	109.00	7,725.91	32.94	2.71	0.68
Concanavalin A affinity chromatography	20	68,320	1.34	51,136.10	2.67	17.92	3.18

The starting material was one kilogram. Enzyme recovery and purification in each step were calculated on the basis of crude extract.

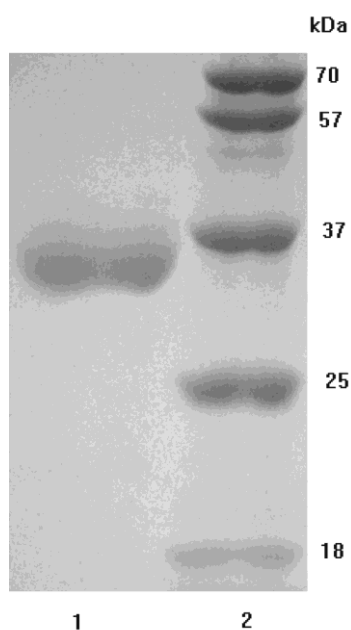
### Optimum pH and effect of temperature

It was known that pH is a key factor for enzyme activity as it changes ionization states of protein and ionization of substrate (Gawlik-Dziki et al., 2008). Some researchers documented that lettuce peroxidase was stable at a wide pH range (Bestwick et al., 1998; Tomás-Barberán et al., 1997) and the optimum pH was reported to be between 6.0 and 8.5 (Onsa et al., 2006). The wide range of optimum pH was due to mixed isoenzymes as peroxidase was not purified or only partially purified. For determining optimum pH of lettuce peroxidase, its activity was assayed at different pH values in the range of 3.0 to 6.5 at intervals of 0.5. The results show that maximal activity was at pH 5.0 (Figure 4), which was lower than 7.0 reported by Altunkaya and Gökmen (2011), suggesting that the purified isoenzyme was not one of isoenzymes reported previously.

To determine optimum temperature of lettuce peroxidase, its activity was assayed after incubation at various temperatures. The optimum temperature was 45°C (Figure 5), which was higher than the optimum temperature of 30°C reported earlier (Altunkaya and Gökmen, 2011). The optimum pH and temperature of peroxidase varies from species of plants. For example, the peroxidase from chick pea showed that optimum pH and temperature were 5.5 and 45°C (Bhatti et al., 2006). The peroxidase from turnip showed the optimums were 6.0 and 50°C (Singh et al., 2002). The peroxidase from cauliflower showed the optimums were 5.0 and 30°C (Köksal and Gülçin, 2008). Interestingly, the earlier reported peroxidase isoenzyme from lettuce showed different optimum pH and temperature (7.0 and 30°C) (Altunkaya and Gökmen, 2011), quite different from this newly isolated isoenzyme (5.0 and 45°C) (Figures 4 and 5). The results indicate that peroxidases differ in optimum



**Figure 2.** Chromatography of lettuce peroxidase on concanavalin A column. The fractions exhibiting peroxidase activity pooled on Sephadex G-100 were added to concanavalin A column. The column was equilibrated with 0.1 M Tris-HCl containing 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub> and 0.5 mM NaCl.



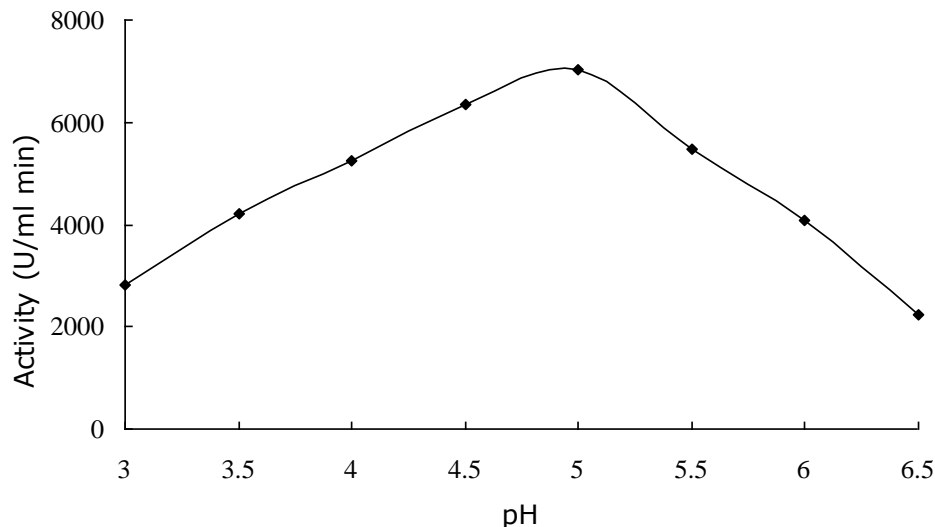
**Figure 3.** SDS-PAGE of lettuce peroxidase. Protein bands were stained with Coomassie blue R-250. Lane 1 peroxidase after concanavalin A chromatography (4 μg protein), Lane 2 protein markers.

pH and temperature even when they are from the same species.

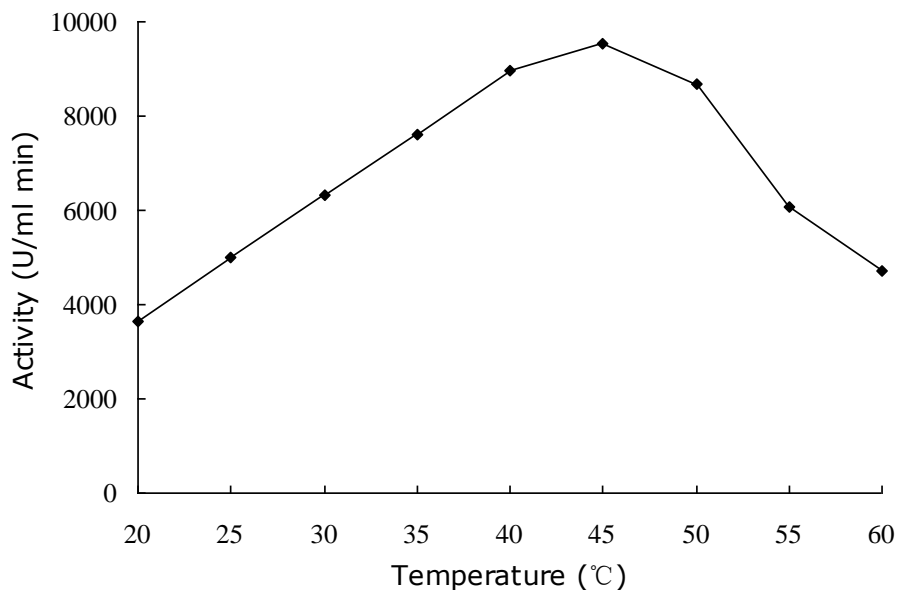
The plant peroxidases are glycosylated proteins and regarded as one of the most heat-stable enzymes (Wang et al., 2009). Peroxidase from lima legumes was stable below 55°C, but rapidly inactivated at 60°C (Wang et al., 2009). Peroxidase from garlic almost totally retained its original activity after incubation at 50°C for two hours (Marzouki et al., 2005). Peroxidase from bitter melon retained nearly 50% of its initial activity after incubation at 60°C for one hour (Fatima and Husain, 2008). For determining thermostability of peroxidase from lettuce, its activity was assayed after incubated at 60°C. After incubation for one hour, it retained 55% of the activity (Figure 6). By contrast, it was less heat stable than some other peroxidases such as peroxidases from soybean and palm, which were stable at the temperature over 80°C (Marzouki et al., 2005). However, this result shows that peroxidase from lettuce is a moderate thermostable enzyme.

### Substrate specificity

To compare substrate specificity,  $K_m$  and  $V_{max}$  were determined with guaiacol, pyrogallol, ABTS and catechol



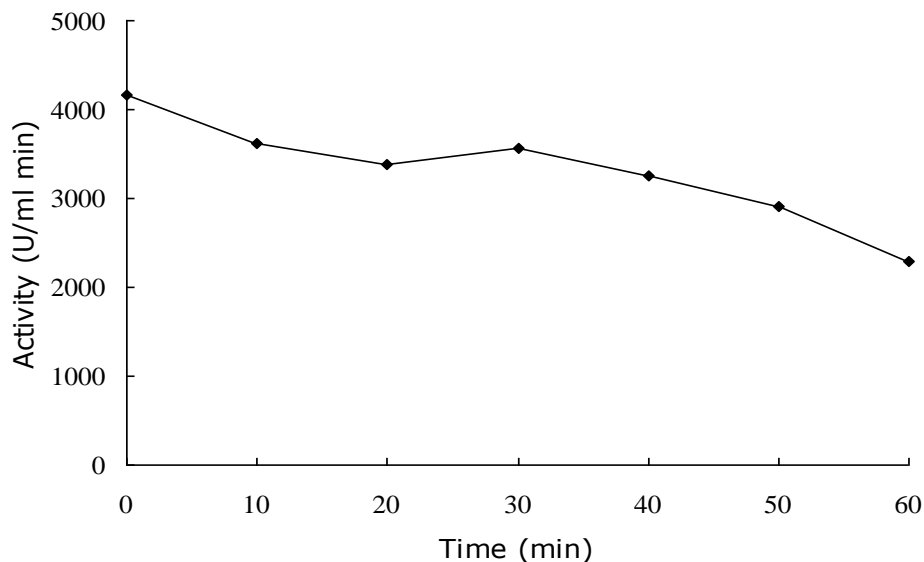
**Figure 4.** Effect of pH on lettuce peroxidase activity. The activity of lettuce peroxidase was determined using guaiacol substrate.



**Figure 5.** Optimum temperature of lettuce peroxidase. The activity of lettuce peroxidase was determined using guaiacol substrate in the temperature range of 20 to 60°C at intervals of 5°C.

substrates. Five different concentrations of substrate were used to assay the enzyme activities while  $H_2O_2$  was constant.  $K_m$  and  $V_{max}$  for the aforementioned four substrates was 4.74, 1.96, 3.75 and 2.95 mM, and 10585, 2824, 1146 and 2302 U/ml min, respectively (Table 2), which showed that pyrogallol substrate was the most affinitive to the enzyme while  $K_m$  for guaiacol was higher than the others. This result was similar to the peroxidase from cauliflower buds, but the peroxidase

from cauliflower had much larger  $K_m$  for guaiacol (141.61 mM) than the other substrates (Köksal and Gülçin, 2008). The peroxidase from bitter melon also had a higher  $K_m$  for guaiacol (5.2 mM) than pyrogallol (5.0 mM), but its  $K_{cat}$  for guaiacol was much higher (Fatima and Husain, 2008). For peroxidase from lettuce, although it had  $K_m$  value of 4.74 mM for guaiacol, the  $V_{max}$  for guaiacol reached at 10585 U/ml min, indicating that guaiacol substrate is suitable for this enzyme.



**Figure 6.** Effect of temperature on lettuce peroxidase incubated at 60°C. The activity of lettuce peroxidase was determined using guaiacol substrate.

**Table 2.** Kinetic parameters of peroxidase with different substrates.

Substrate	$K_m$ (mM)	$V_{max}$ (U/ml min)
Guaiacol	4.74	10585
Pyrogallol	1.96	2824
ABTS	3.75	1145
Catechol	2.95	2302

$K_m$  and  $V_{max}$  were determined from Lineweaver-Burk plot. ABTS, 2, 2'-azino-bis-(3-ethylbenzthiazolin-6-sulfonate).

**Table 3.** Effect of organic solvents and metal ions on lettuce peroxidase.

Parameter	Relative activity			
	2%	5%	15%	25%
<b>Organic solvents</b>				
Propylene glycol	95.44	85.77	77.56	63.50
Methyl alcohol	71.53	50.00	46.72	30.11
Ethanol	63.87	60.58	57.66	55.84
Acetone	62.41	61.13	60.77	58.39
<b>Metallic ions</b>	<b>1 mM</b>	<b>5 mM</b>		
Fe <sup>3+</sup>	71.00	66.23		
Zn <sup>2+</sup>	66.72	51.26		
Ca <sup>2+</sup>	46.71	36.82		
Cu <sup>2+</sup>	43.57	42.42		
Mn <sup>2+</sup>	47.20	44.15		
Mo <sup>6+</sup>	90.12	67.55		

### Effect of organic solvents and metal ions

It was reported that the peroxidase from chick pea was slightly inhibited by Zn<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>, Al<sup>3+</sup> and Hg<sup>2+</sup>

at concentration of 1 mM, while Ca<sup>2+</sup> and Mg<sup>2+</sup> did not inhibit the enzyme activity (Bhatti et al., 2006). The peroxidase from bitter melon was not inhibited by Mn<sup>2+</sup> and Co<sup>2+</sup> even when metal ions were at a very high

concentration of 9 mM (Fatima and Husain, 2008). As shown in Table 3, the effects of various inhibitors were determined using guaiacol substrate. The peroxidase from lettuce was inhibited by all inhibitors. The enzyme lost over 40% activity in 25% concentration of methyl alcohol, ethanol and acetone, and it was apparently inhibited by metal ions ( $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Mn}^{2+}$ ) with the concentrations of 1 mM and 5 mM (Table 3). This result is similar to that reported by Marzouki et al. (2005), that is, peroxidase from garlic, also inhibited by metal ions and organic solvents such as  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ , acetone and ethanol. The effects of inhibitors on peroxidase activity could be important in use of the raw materials.

## Conclusions

In summary, a peroxidase from lettuce stems was purified to homogeneity in this study. Its molecular mass was 35 kDa. The optimum pH and temperature was 5.0 and 45°C. The enzyme showed moderate thermostability at 60°C. Its  $K_m$  for guaiacol, pyrogallol, ABTS and catechol was 4.74, 1.96, 3.75 and 2.95 mM, respectively. Its activity was inhibited by metal ions ( $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Mn}^{2+}$ ) and organic solvents (methyl alcohol, ethanol and acetone).

## ACKNOWLEDGMENTS

The work was a project supported by Scientific Research Fund of Hunan Provincial Education Department (10A63) and Science and Technology Planning Project of Hunan Province, China (2009FJ3159).

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