Study of physicochemical and kinetics features of peroxidase isolated from hoary cress (Cardaria draba L.)

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Abstract
New peroxidase may be versatile was investigated in different parts of hoary cress. Roots regarded as a rich source of enzyme (2095.23 U mg\(^{-1}\)) comparison in other botanical parts. Peroxidase was purified from roots by ammonium sulfate, dialysis and Sephadex G-100 gel filtration, showed final degree of purity and recovery 2.70 and 54.11% respectively. Molecular mass, optimum of pH, temperature and time of enzymatic reaction were 56.234 kDa, 6.5, 40\(^{\circ}\)C, 3 min. respectively. \(K_m\) and \(V_{\text{max}}\) were estimated of each substrate (guaiacol and hydrogen peroxide), noticed high affinity to hydrogen peroxide. Competitive sodium azide inhibitor was suppressed peroxidase totally at 90 mM.

Introduction
Peroxidases (E.C.1.11.1.7) belong to the oxidoreductases class have heme III, protoporphyrin IX and ferriprotoporphyrin IX as prosthetic group (Hamid and Rehman, 2009). Members of these groups catalyze the oxidation number of substances in the presence of hydrogen peroxide (Köksal and Gülçin, 2008). Peroxidases exist in very wide range of the plants especially at vacuoles, tonoplast, plasmalemma and inside and outside the cell wall (Vitali et al., 1998). In addition present in another living organisms such as animal and micro-organisms (Chan et al., 2002). Different physiological functions of these enzymes reflect the presence as isoenzymes in plant cell organelles. They are include plant hormone regulation (Gutierrez et al., 2009), participated in lignifications and suberization (Musel et al., 1997; Quiroga et al., 2000), protection toward \(H_2O_2\) and other oxidants (Martinez et al., 1998), defense properties against pathogenic causes (Chittoor et al., 1999) and have role in tolerance of drought in plant (Yamauchi and Minamide, 1985). According to previous studies peroxidases used in varied scientific fields involve biotechnology, biochemistry, clinical and industrial purposes. It used in diagnosis and biosensors for recombination and expression of recombinant protein and protein engineering (Ryan et al., 2006). Immunochemistry and ELASA (Sanchez et al., 1997). Manufacturing of many aromatic complexes, elimination of phenolics complexes from waste water and peroxides from foods, beverage and industrial wastes (Torres et al., 1997). This paper deal with the found new source of peroxidase can be utilized in different fields that mentioned as above.

Materials and methods
Materials
\(H_2O_2,\) Guaiacol, Sodium phosphate monobasic, Sodium phosphate dibasic, Citric acid, Sodium citrate, Acetic acid, Sodium acetate. Tris-base, HCl. Sodium carbonate, Sodium bicarbonate, BSA, Copper sulfte, Sodium carbonate, Sodium hydroxide, Folin-ciocalteu phenol reagent, Ammonium sulfate, Sephadex G-100, Dextran, Insulin, Lysozyme, Ovalbumin, Sodium azide

Methods
Protein content

Protein concentration in plant material estimated by folin method was described by Lowery, (1951) using of BSA as standard protein.

Extraction of crude peroxidase

Whole plants were harvested from its nature habitat (Imam Aon village, south of Hilla city) and identified as Cardaria draba by Dr. Abdulkareem Al-Bermani, in department of biology, college of science for girls, Babylon University. Different parts (roots, stems, leaves and fruits with its seeds) were selected for Cardaria draba peroxidase (CDP) extraction according to Zia et al. (2001). Plant materials were rinsed by distilled water and submerged overnight in distilled water. Homogenization of 100g in blender with 400 ml distilled water for 15 min and then centrifuged at 10,000 rpm tor 15 min at 4°C. Peroxidase (supernatant) was filtered for remove the debris and then warmed at 65°C by water bath for 3 min for catalase suppression. The extract was cooled immediately by placing in ice bath for 10 min. then used for determination of peroxidase activity and protein concentration.

Peroxidase activity

CDP activity was determined according to Gülçin et al., (2005). Twenty five μl of crude peroxidase extract was added to a solution of 1 ml 22.5 mM H$_2$O$_2$ and 1 ml 45 mM guaiacol. 0.1 M phosphate buffer pH 7.0 was added to solution for obtain final volume (3 ml). The enzyme activity was assayed by the change of the absorbance (0.01) at 470nm wavelength monitored for 3 min at 20°C.

Purification

Ammonium sulfate (NH$_4$)$_2$SO$_4$ fractionation

First step of CDP purification is precipitation by (NH$_4$)$_2$SO$_4$, crude extracts fractionated independently by solid ammonium sulfate to reach final concentrations of 30, 40, 50, 60, 70 and 80%. The precipitate obtained after centrifugation (15 min at 10000 rpm and 4°C) re-suspended in 0.1 M phosphate buffer pH 7.0.

Dialysis

Second step of CDP purification is dialyzed of concentrated solution with highest specific activity against 0.05 M phosphate buffer pH 7.0 (24hr at 4°C) for salts removing.

Sephadex G-100 gel filtration

The dialyzed sample of CDP was poured on the 1×50 cm of Sephadex G100 column previously equilibrated by 0.2 M phosphate buffer pH 7.0. The flow rate of eluted buffer was adjusted approximately at 30 ml/h and fractions of 3 ml were collected for detection of absorbance and activity.

Physicochemical features

Molecular mass

Sephadex G100 column pre-calibrated with blue dextran and different standard protein involved insulin, lysozyme, ovalbumin and BSA used to calculate CDP molecular mass. The purified peroxidase re-chromatographed, molecular mass was determined by $V_e/V_o$ vs log molecular mass plot of standard proteins.
Optimal reaction pH
Various buffers with wide values of pH (3-10.5) were used at 0.5 interval included citrate, acetate, phosphate, Tris-HCl and carbonate-bicarbonate buffer.

Optimal reaction temperature
Peroxidase activity was estimated with different temperatures (10-70) at 5°C interval.

Optimal reaction time
Varied times (1-5) at 1 minute interval used to determine of peroxidase activity.

Kinetics
Enzyme activity towards various concentrations of guaiacol and H\(_2\)O\(_2\) were selected to determination of the Michaelis-Menten constant \(K_m\) and apparent \(V_{max}\) using the method of Lineaweaver-Burk under optimum pH and temperature conditions.

Inhibition
Variable concentration of sodium azide (10-100) at interval 10 mM were used to study of inhibitory effect on CDP activity.

Results and discussion
Peroxidase activity was differed in all studied homogenized parts of the plant, but the highest specific activity (2095.23 U mg\(^{-1}\)) was measured in roots compared with other parts (Figure 1). Homogenization in buffer used in easily extracted of peroxidase from plant material. Peroxidase liberates from the destroyed cells into the buffer then separated from the cells ruins by filtration or centrifugation. To detect of the peroxidase activity, the crude extract mixed with peroxide and guaiacol, the H\(_2\)O\(_2\) is immediately converted to H\(_2\)O and O\(_2\) when peroxidase is present. The O\(_2\) reacts with the guaiacol produced a brown color (oxidized guaiacol) can be assayed by spectrophotometer (Bynum, 1996). Increasing of the activity in the roots may be reflect the relationship of our investigated plant with other species belong to same family (Brassicaceae). These plants such as horseradish (Armoracia rusticana) and Turkish black radish (Raphanus sativus) have roots with richest sources of peroxidases (Veitch, 2004; Şişcioğlu et al., 2010). Other parts used as source for extraction and purification of peroxidase (see below).
Ammonium sulfate 70% saturation before and after dialysis raised the specific activity of CDP to 3943.17 and 4200.5 unit mg$^{-1}$ respectively (Table 1). Fractionation by ammonium sulfates increasing the concentration of the salt resulting in peroxidase agglomeration and become insoluble without absence of enzyme activity. In addition solubility does not depending on temperature that facility of peroxidase separation from other molecules and proteins. Dialysis is associated step with ammonium sulfate precipitation used to elimination of salts and different small molecules from active fraction (Allen, 2003). Crude extract commonly treated by ammonium sulfate as first step of peroxidase purification with some exceptions such as fractionation by acetone of cabbage (Brassica oleracea capitata L.) leaves peroxidase(Yazdi et al., 2002). However crude enzyme from turnip, tomato, radish, horseradish legumes and roots was 50-85% saturated by (NH$_4$)$_2$SO$_4$ (Rehman et al., 1999) while filtered juice of turnip (Brassica rapa) roots was precipitated by solid ammonium sulfate up to 75% saturation (Hamed et al., 2009). Solid salts also was added to crude extract of Soybean seeds until it become 50% saturated then supernatant was adjusted to 85% (Habib et al., 2003).

Table 1 Steps of CDP purification

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Activity (U ml$^{-1}$)</th>
<th>Protein (mg ml$^{-1}$)</th>
<th>Specific activity (U mg$^{-1}$)</th>
<th>Total activity (U)</th>
<th>Degree of purity</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>25</td>
<td>440.02</td>
<td>0.21</td>
<td>2095.33</td>
<td>11000.5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>70% (NH$_4$)$_2$SO$_4$ fractionation</td>
<td>10</td>
<td>670.34</td>
<td>0.17</td>
<td>3943.17</td>
<td>6703.4</td>
<td>1.88</td>
<td>60.93</td>
</tr>
<tr>
<td>Dialysis</td>
<td>12</td>
<td>588.07</td>
<td>0.14</td>
<td>4200.5</td>
<td>7056.84</td>
<td>2</td>
<td>64.15</td>
</tr>
<tr>
<td>Sephadex G100</td>
<td>15</td>
<td>396.83</td>
<td>0.07</td>
<td>5669</td>
<td>5952.45</td>
<td>2.70</td>
<td>54.11</td>
</tr>
</tbody>
</table>

After dialysis solution having maximum activity was loaded on Sephadex G-100 gel filtration, degree of purity and recovery of active peak at this step were 2.70 and 54.11% respectively (Figure 2 and Table 1). Two reasons to select the gel filtration directly without passing with ion exchange step, firstly: Relatively low concentration of proteins at dialysis step.
(0.14 mg ml\(^{-1}\)) that not need further purification steps, secondly unknown the net charge of CDP solution although the isoelectric point of horseradish peroxidase is 7.2 (Xialing and Lin, 2009). Reducing of purification step especially when recovery was more than 64% in dialysis step may be benefit for cost lowering if CDP exploited in biotechnology or industrial opportunity. As follow some types of Sephadex were advantaged in peroxidase purification because their fast separation of high and low molecular mass enzyme of desalting sample (Wu, 1995). Sephdex G100 and 75 used to increasing purity of peroxidase from peach fruit (Neves, 2002), apple and orange seeds (Zia et al., 2011) as last step of purification.

Figure 2 Sephadex G100 column (1×50) cm of CDP pre-equilibrated by 0.2 M phosphate buffer pH 7.0. The flow approximately was 30 ml/h and 3 ml for each fraction.

Graph presented as a figure 3 showed \(V_e/V_o\) value of CDP was 6.4 versus 4.75 log of molecular mass, antilog of last value is 56.234 kDa that represented molecular weight of CDP. This graph and SDS-PAGE electrophoresis used to detection molecular mass of plant peroxidases were located between 28-60 kDa reviewed by Hiraga et al. (2001). Unlike that more recent study reveled clarified peroxidase from Brassica Oleracea has molecular mass of 95 kDa (Gülçin and Yildirim, 2005).
The optimum pH for CDP activity was 6.5 (Figure 4), i.e. suggested as acidic peroxides. There are three types of peroxidase identify according to their pH activity; acidic, neutral and alkaline (Sakkarov, 2001; Yadav et al., 2011). The optimum temperature for CDP was determined to be 40°C (Figure 5). Varied optimum pH and temperature of studied peroxidase activity compared with other plant peroxidase reported in many articles belong to substrate types used in enzymatic reactions. Şişcioğlu et al. (2010) showed changing in optimum pH and temperature according to substrates were used such as guaiacol, ABTS, catechol, pyrogallol and 4-methyl catechol even peroxidase purified from same species.
Increasing of CDP activity gradually until optimum time observed at 3 min, then decreasing at 4 and 5 min respectively (Figure 6). Typically substrate is a time exhausting depending (Marangoni, 2003), subsequently lowering of activity occurring when substrate consumed with time progress.

The double-reciprocal plot under optimal conditions used to determine the ability of catalytic oxidation of guaiacol (reducing agent) by H$_2$O$_2$ in the presence of CDP. $K_m$ and $V_{max}$ were $0.15 \times 10^3$ mM and $0.55 \times 10^3$ mM min$^{-1}$ respectively when guaiacol used as variable substrate concentrations at the fixing concentration of H$_2$O$_2$ (Figure 7). On the other hand same kinetic parameters were $0.02 \times 10^3$ mM and $0.25 \times 10^3$ mM min$^{-1}$ when H$_2$O$_2$ used as different concentrations at the constant concentration of guaiacol (Figure 8). The low $K_m$ of H$_2$O$_2$ compared with guaiacol in present work refer to greater affinity of CDP for H$_2$O$_2$ and corresponding with results of Vernwal et al. (2006), they calculated $K_m$ value for guaiacol and H$_2$O$_2$ were 6.5 mM and 0.33 mM respectively of Solanum melongena fruit juice peroxidase. Şat (2008) indicated to conflicting results that showed higher affinity for guaiacol of peroxidase from Jerusalem artichoke (Helianthus tuberosus L.) tubers. Decreasing of $K_m$ parameter for hydrogen peroxide pointed out an increasing number of hydrogen molecule or hydrophobic
interactions between the iron group in the active site of peroxidase and the substrate (Onsa et al., 2006).

Figure 7 Michaelis-Menten and maximal velocity of CDP by Lineweaver-Burk plots for guaiacol

Figure 8 Michaelis-Menten and maximal velocity of CDP by Lineweaver-Burk plots for hydrogen peroxide

Sodium azide concentrations for inhibition of CDP illustrated in figure 9 showed dropping of activity about 52% at 30 mM whereas complete inhibition appeared at 90 mM. The inhibition gives evidence of presence of iron at active site because sodium azide is competitive and belong to metalloenzyme an inhibitor (Sugumaran, 1995). The effect of sodium azide on peroxidase activity studied by Vernwal et al. (2006) and Yadav et al., 2011. The results of these studies showed decline of activity to half at 20 mM while activity completely inhibited at 100 mM. Other article on Gorgonia ventalina pointed out 1 mM and 10 mM sodium azide inhibited peroxidase activity by 52±8% and 85±1.8% respectively (Mydlarz, and. Harvell, 2006).
Figure 9 Inhibition of CDP activity by sodium azide.

References


(\textit{Cardaria draba} L.)

دراسة الخواص الفيزيوكيميائية والحركية لانزيم للبروكسيديز المعزول من نبات الكنيبرة

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تم التحري عن مصدر جديد للبروكسيديز في أجزاء مختلفة من نبات الكنيبرة ربما يكون ذو استخدامات متعددة، حيث عدت الجذور المصدر الأغنيي بالبروكسيديز مقارنة بالأجزاء النباتية الأخرى وفعالية نوعية قدرها 2395.23 وحدة/ملغم. نقي البروكسيديز من الجذور بواسطة الترسيب بكبريتات الأمونيوم، الديازة والترشيح الهلامي (سفادكس 100-G) إذ كانت درجة النقاوة والخصائص النهائية 70 و 54.11 على التوالي. الوزن الزيتي والدهني، درجة الحرارة والزمن الأمثل للتفاعل الإنزيدي كان 6.5د.م. ال 6.5 درجة مئوية، 40 دقيقة نتائج. فعالية كل من ثابت ميكاس منتن والسرعة القصوى باستخدام الكوكلوزون و بروكسيد الأوليدز كمادتي نفاعل للإنزيم حيث لوحظ الفئة عالية للإنزيم تجاوب بروكسيد الأوليدز كمادتي نفاعل ثم النيازيدون، ثبت النيازيدون كمادتي نفاعل بواسطة المثبط التنافسي sodium azide وتركيز 90 ملي مولر.