Study of physicochemical and kinetics features of peroxidase isolated fromhoary 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^{o}C, 3 min. respectively. K_m and V_{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 (Müsel *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 materialswere 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_2O_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^{o}C.

Purification

Ammonium sulfate $(NH_4)_2SO_4$ fractionation

First step of CDP purification is precipitation by $(NH_4)_2SO_4$, crude extracts fractionated independently by solid ammonium sulfate to reach final concentrations of 30, 40, 50, 60, 70and 80%. The precipitate obtained after centrifugation (15 min at 10000 rpm and 4^{o}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^{o}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 equilibratedby 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^{o}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_2O_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 (Figure1). 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_2O_2 is immediately converted to H_2O 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*) andTurkish 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).

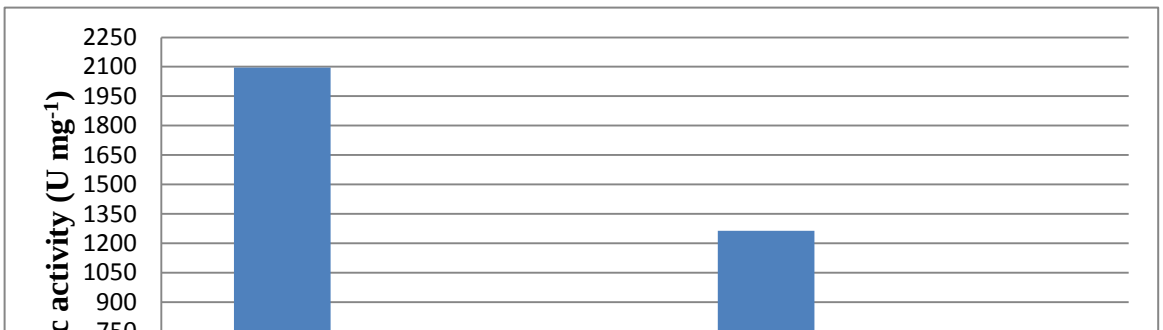

Figure 1 Specific activity of CDP in different botanic parts

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 $(NH_4)_2SO_4$ (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

Step	Volume (ml)	Activity (U ml^{-1})	Protein (mg ml^{-})	Specific activity(Umg^{1})	Total activity(U)	Degree of purity	Recovery (%)
Crude homogenate	25	440.02	0.21	2095.33	11000.5	1	100
70% $(NH_4)_2SO_4$ fractionation	10	670.34	0.17	3943.17	6703.4	1.88	60.93
Dialysis	12	588.07	0.14	4200.5	7056.84	2	64.15
Sephadex G100	15	396.83	0.07	5669	5952.45	2.70	54.11

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.

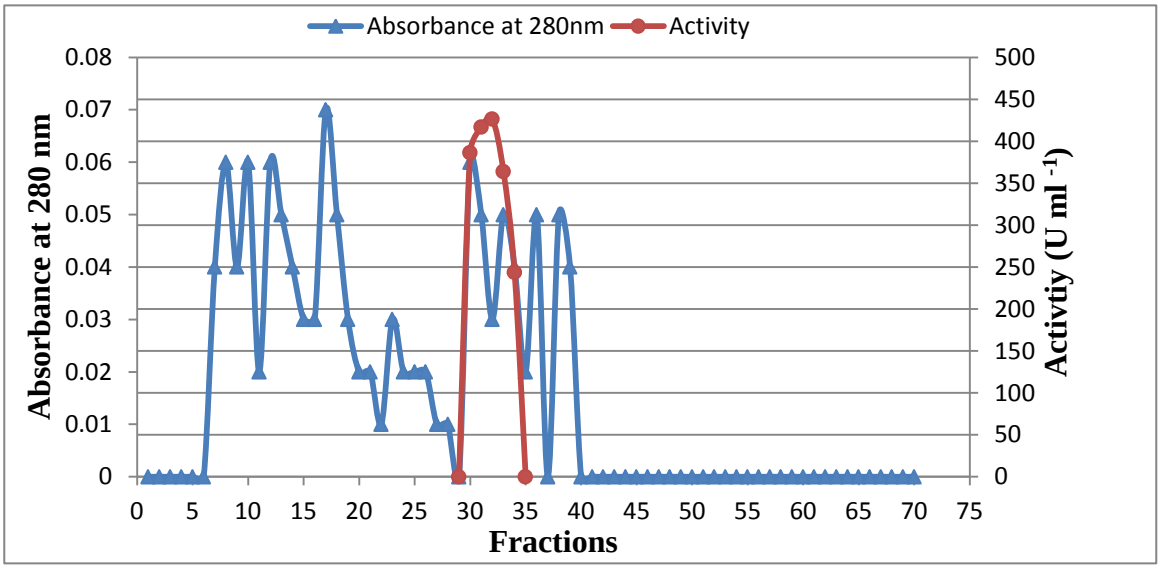

Figure 2 Sephadex G100 column (1×50) cm of CDP pre-equilibratedby 0.2 M phosphate buffer pH 7.0. The flow approximately was 30 ml/h and 3 ml for each fraction.

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).

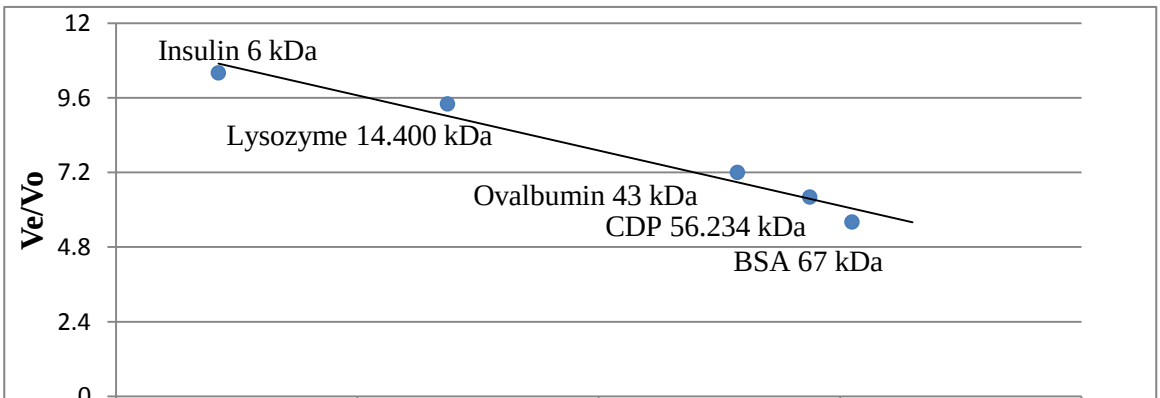

Figure 3 Molecular weight of CDP

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^{o}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.

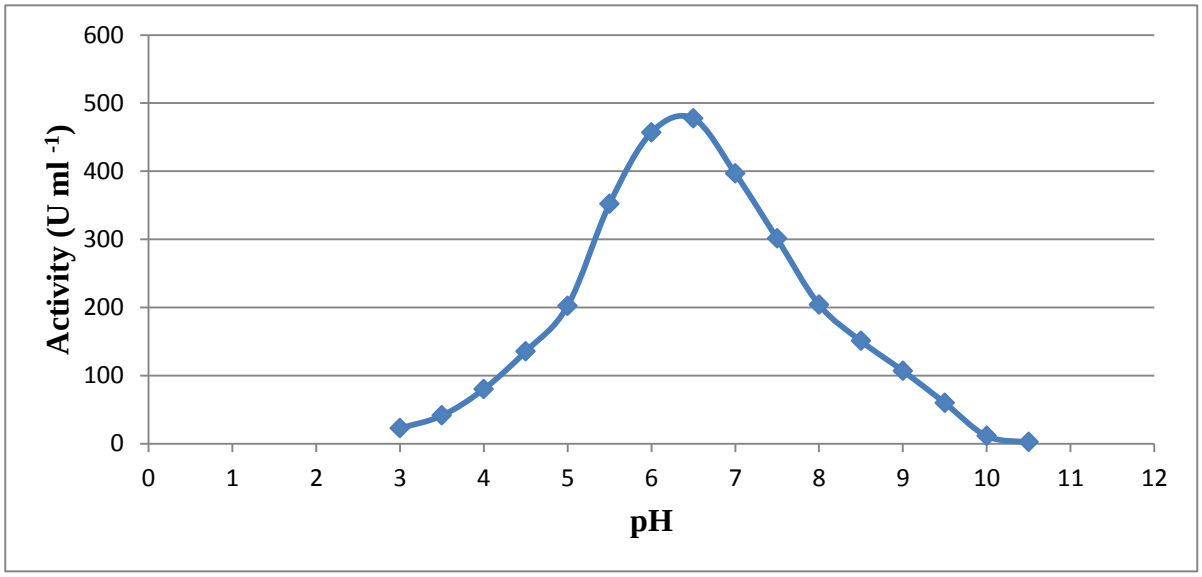

Figure 4 Optimum pH of CDP

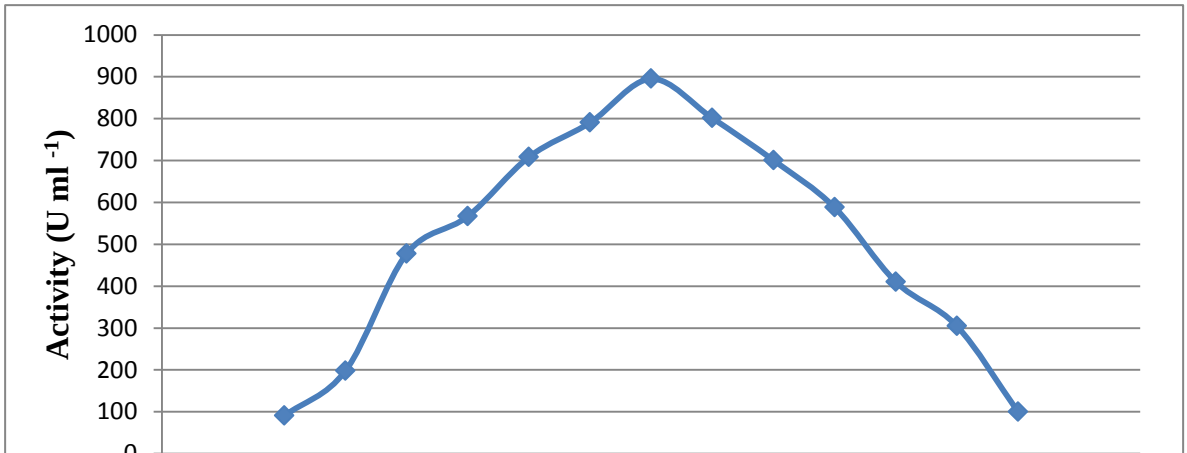

Figure 5 Optimum temperature of CDP

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.

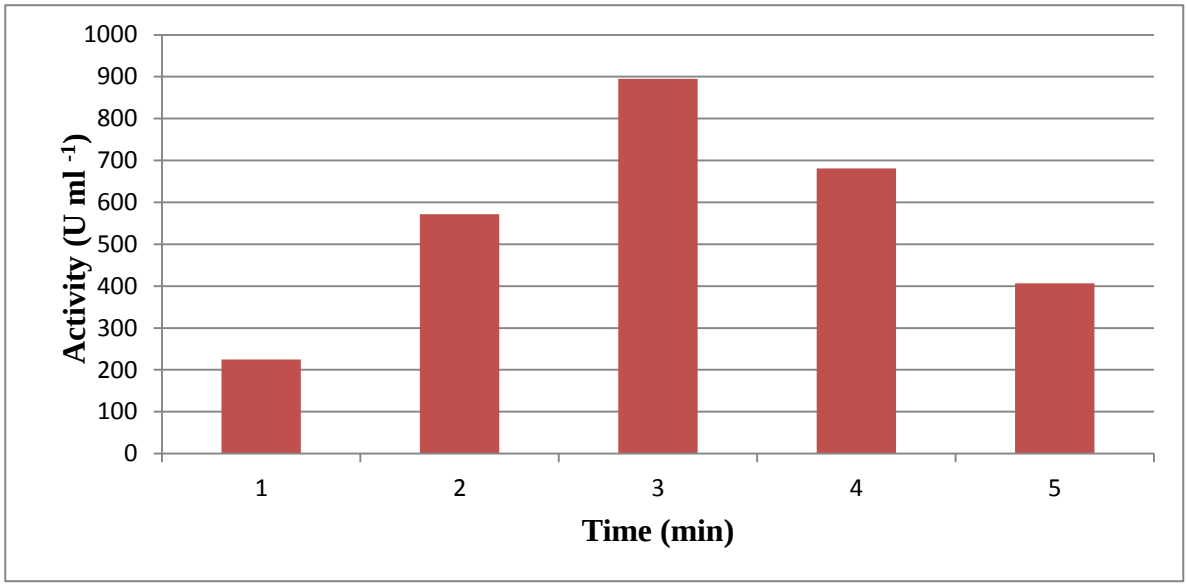

Figure 6 Optimum time of CDP activity

The double-reciprocal plot under optimal conditions used to determine the ability of catalytic oxidation of guaiacol (reducing agent) by H_2O_2 in the presence of CDP. K_m and V_{max}were 0.15×10^3 mM and 0.55×10^3 mM min^{-1} respectively when guaiacol used as variable substrate concentrations at the fixing concentration of H_2O_2 (Figure 7). On the other hand same kinetic parameters were 0.02×10^3 mM and 0.25×10^3 mM min^{-1} when H_2O_2 used as different concentrations at the constant concentration of guaiacol (Figure 8). The low $K_m$of H_2O_2 compared with guaiacol in present work refer to greater affinity of CDP for H_2O_2 and corresponding with results of Vernwal *et al*. (2006), they calculated K_m value for guaiacol and H_2O_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).

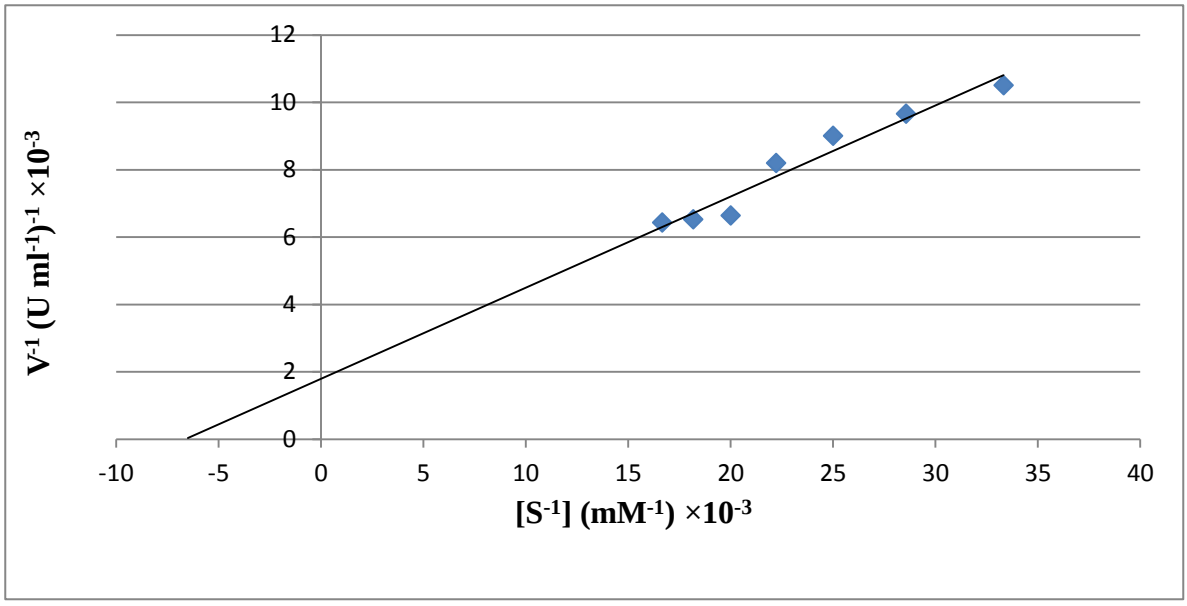

Figure 7 Michaelis-Menten and maximal velocity of CDP by Lineweaver-Burk plots for guaiacol

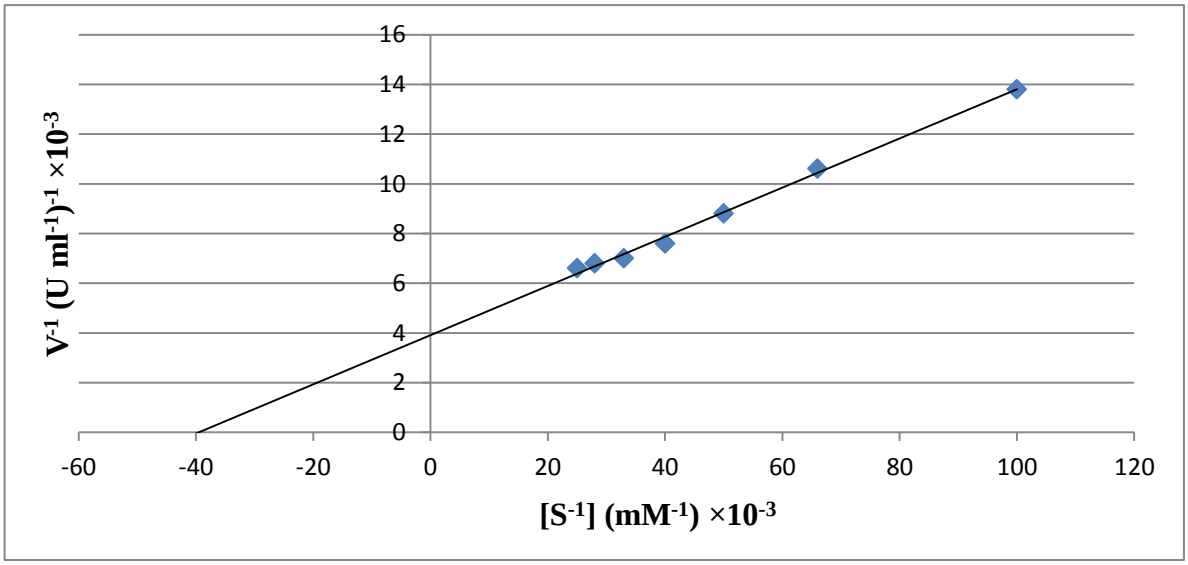

Figure 8 Michaelis-Menten and maximal velocity of CDP by Lineweaver-Burk plots for hydrogen peroxide

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 (Sugumaran1995). 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).

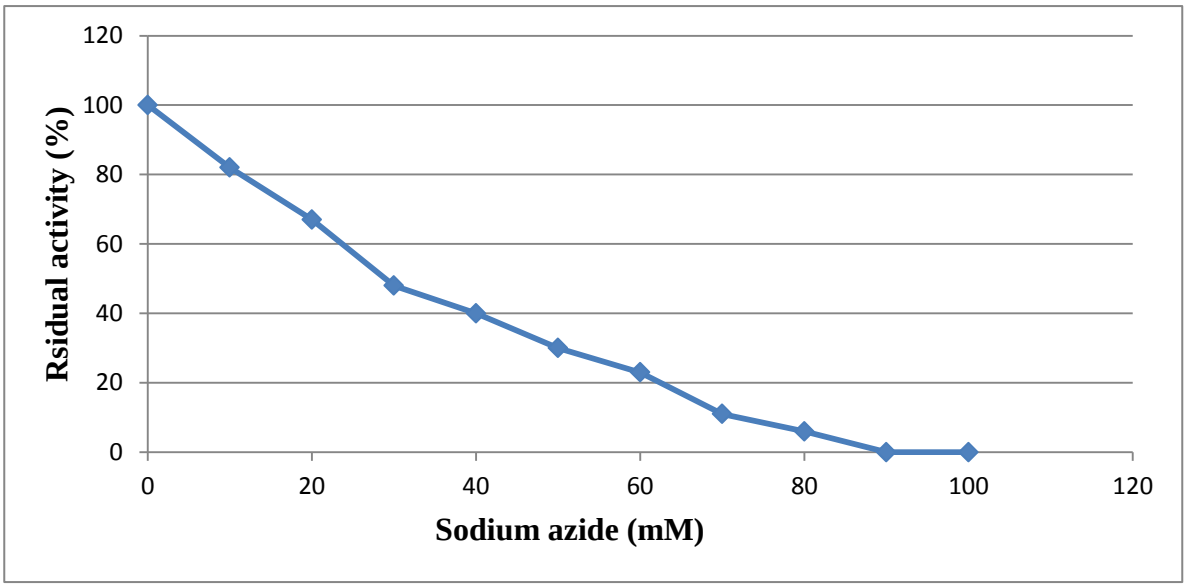

Figure 9 Inhibition of CDP activity by sodium azide.

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دراسة الخواص الفيزوكيميائية والحركية لانزيم للبروكسيديز المعزول من نبات الكنيبرة (*Cardaria draba* L.)

نزار عبدالأمير حمزة / جامعة القادسية/ كلية العلوم/ قسم علوم الحياة

تم التحري عن مصدر جديد للبروكسديز في اجزاء مختلفة من نبات الكنيبرة ربما يكون ذو استخدامات متعددة، حيث عدت الجذور المصدر الاغنى بالانزيم مقارنة بالاجزاء النباتية الاخرى وبفعالية نوعية قدرها2095.23 وحدة/ملغم. نقي البروكسيديز من الجذور بواسطة الترسيب بكبريتات الامونيوم، الديلزة والترشيح الهلامي (سفادكس G-100) اذ كانت درجة النقاوة والحصيلة النهائية 2.70 و 54.11 على التوالي. الوزن الجزئيي ، الاس الهايدروجيني ودرحة الحرارة والزمن الامثل للتفاعل الانزيمي كان 56.23 كيلو دالتون، 6.5، 40 درجة مئوية و 3 دقائق على التوالي. قيس كل من ثابت ميكالس منتن والسرعة القصوى باستخدام الكويكول و بيروكسد الهايدروجين كمادتي تفاعل للانزيم حيث لوحظ الفة عالية للانزيم تجاة بيروكسد الهايدروجين. ثبط البروكسيديزكليا بواسطة المثبط التنافسي sodium azide وبتركيز 90 ملي مولر.