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Purification and Biochemical Properties of Peroxidase from *Brassica rapa* cv. Iraqi turnip

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Peroxidase from Brassica rapa cv. Iraqi turnip germinated seeds was selected as a good starting enzyme from 22 cultivars of Cruciferae family cultivated in Egypt. Iraqi turnip peroxidase was purified by using ammonium sulphate precipitation, chromatography on Phenyl Sepharose and Sephacryl S-200. Four peroxidase isozymes (PODI to PODIV) have been separated from Phenyl Sepharose. PODII with the highest specific activity was applied on a Sephacryl S-200. The purity of Sephacryl S-200 PODII was confirmed by polyacrylamide gel electrophoresis and its specific activity is 5333 units/mg protein which represented 11.6 fold purification over the crude extract with 25% recovery and Reinheit Zahl (RZ value equal 1.4. The molecular weight of PODII is 32 kDa by using gel filtration and SDS-PAGE suggesting that a monomeric structure for PODII. The substrate specificity of PODII was studied by using a number of chromogenic substrates, where its affinity decreasing in the order of guaiacol > o-dianisidine > 4-aminoantipyrene with K_m values of 13.6, 16, 25 mM, respectively. The enzyme had pH and temperature optima at 5.5 and 40° C, respectively. PODII was stable up to 30 °C. The effectiveness of metal cations as inhibitors for PODII was Hg^{2+} >Mn^{2+}>Ni^{2+} > Ba^{2+} >Co^{2+} >Ca^{2+}, Cu^{2+} >Mg^{2+}. Kinetic study of inhibition showed that PODII was competitively inhibited by SDS, EDTA and DTT with inhibition constant K_i of 3.4, 3.9, and 4.7, respectively. In conclusion, Iraqi turnip PODII could be potentially used for bioremediation of phenolic compounds polluted the environment.

Keywords: *Cruciferae; Brassica rapa; Iraqi turnip; peroxidase; enzyme purification; enzyme properties.*

1. INTRODUCTION

Peroxidases (EC 1.11.1.7) catalyze the oxidation of a wide variety of substrates (aromatic compounds, heteroatom compounds, epoxidation, and the enantioselective reduction of racemic hydroperoxides using H_2O_2 or other peroxides [1]. Peroxidase is probably the well-suited enzyme for the preparation of enzyme-conjugated antibodies which are used in enzyme linked immunosorbent assay due to its ability to yield chromogenic products at low concentrations. This enzyme has been widely used as an indicator of the adequacy of vegetables blanching due to its relatively high thermal stability and wide distribution [2]. Currently, peroxidases are used also in organic synthesis for production of polymers and for biotransformation of various drugs and chemicals. Peroxidase enzymes span the bioscience and biotechnology spectra, ranging from bioremediation and biocatalysis through diagnostics and biosensors to recombinant protein expression, transgenics, bioinformatics, protein engineering and even to therapeutics [3, 4].

Peroxidases have been isolated from several plant sources such as tomato [5], cabbage [6], melon [7], turnip [8], and horseradish [9]. One of the important features of peroxidases is that they are associated with cell elongation processes and lignin biosynthetic pathway [10]. Plants exposed to acute stress are known to up-regulate their overall peroxidase activity. A possible function of peroxidases in treatment of heavy metals is their contribution in accumulating plants [11]. The peroxidase upregulation as a response of the plant

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to pollutants can already be used for the phytomonitoring of industrial. Peroxidases have been shown to be quite sensitive to atmospheric pollution [12]. Reactive oxygen species such as O_2^- have been shown to be involved in the induction and development of the senescence stage. The superoxide radical O_2^- could be generated by extracellular peroxidase activity following salicylic acid treatment and could act on the senescence induction pathway. The implication of peroxidases is related to the fine balance between cell wall loosening and stiffening [13].

Very little information had been reported about the production of peroxidase from germinated seeds. Therefore, the important goal of this study is to search for preparation of peroxidase from germinated seeds of *Curciferae* family apart from horseradish which produced the main commercial peroxidase in the world. Purification and characterization of peroxidase from Iraqi turnip which contained the highest level of the enzyme among the examined species and cultivars were also studied.

2. MATERIALS AND METHODS

2.1. Plant Materials

Seeds of twenty two cultivars belonging to three genera of *Cruciferae* family (*Brassica, Raphanus, Eurca*) chiefly representing different varieties cultivated in Egypt were examined for the activity of peroxidase. All seeds were obtained from Faculty of Agriculture-Kafr-Elshaikh University, Kafr-Elshaikh, Egypt.

2.2. Germination Condition of Seeds

All seeds of twenty two cultivars selected were surface sterilized with 0.25% (V/V) sodium hypochlorite solution for 5 minutes, washed several times with sterilized water. The seeds were germinated on a piece of moistened cotton in Petri-dishes, and incubated at room temperature in the dark. Germinated seeds were harvested when the seedlings had developed to a stage at which the coleoptiles had expanded to 5-7 cm, before the first true leaf had appeared. After germination of seeds, the sprout and the rootlets were excised and the germinating seed stored at -20 °C until required for analysis.

2.3. Preparation of Crude Extracts

Crude extracts were prepared by homogenization of germinated seeds in mortar using 100 mM sodium acetate buffer, pH 5.6 for 5 min. and centrifuged for 15 min at 12,000 x g. The supernatant was retained and the precipitate was re-extracted with the same buffer and centrifuged for 15 min at 12,000 x g. The two supernatants were pooled and designed as crude extract.

2.4. Peroxidase Assay

Peroxidase activity was carried out according to Lu and Whitaker [14]. The reaction mixture contains 4 mM H_2O_2, 20 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5 and 0.2 unit of enzyme in 1.0 ml final volume. The change of absorbance at 470 nm due to guaiacol oxidation was followed at 30 second intervals. One unit of peroxidase activity is defined as the amount of enzyme which increases the absorbance a value of 1.0 per min under standard assay conditions.

2.5. Purification of Iraqi Turnip Peroxidase

Unless otherwise stated, all steps were performed at 4 - 7 °C using 50 mM sodium acetate buffer, pH 5.5. The crude extract was precipitated by solid ammonium sulphate up to 70% saturation. The precipitate was collected by centrifugation at 12,000 x g for 20 min and dissolved in a least volume of the buffer and dialyzed against the buffer containing 1 M ammonium sulphate. The dialyzate was applied directly to a Phenyl Sepharose CL-4B column (6 x 2 cm) equilibrated with the buffer containing 1 M ammonium sulphate. The exchanged material was eluted with a stepwise gradient of ammonium sulphate prepared in the buffer ranging from 1.0 to 0.0 M at a flow rate of 30 ml/h and 3 ml fractions. Protein fractions exhibiting peroxidase activity were pooled in four peaks (PODI to PODIV) according to their elution order. PODII with highest peroxidase activity was applied to a Sephacryl S-200 column (93 x 1.6 cm) previously equilibrated with the buffer and developed at a flow rate of 20 ml/h and 3 ml fractions.

2.6. Determination of R Z Value

The purity number (Reinheit Zahl (RZ) value) is the ratio of absorbance at 405 to 280 nm. It measures of

the heme content in peroxidase against aromatic acid contents of protein as reference [15].

2.7. Protein Determination and Polyacrylamide Gel Electrophoresis (PAGE)

Protein was determined either by measuring the absorbance at 280 nm [16] or by the method of Bradford [17] using bovine serum albumin as a standard. Electrophoresis under nondenaturing conditions was performed in 10% (w/v) acrylamide slab gel according to the method of Davis [18] using a Tris-glycine buffer, pH 8.3. Protein bands were located by staining with Coomassie Brilliant Blue R-250.

2.8. Molecular Weight Determination

The native molecular weight was determined by gel fitration using Sephacryl S-200. The column (93 x 1.6 cm) was calibrated with cytochrome *C* (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and α-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (V_o).

The subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis [19]. Standard molecular weight: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin dehydrogenase (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000) and α-lactalbumin (14,200) were used for for the calibration curve.

2.9. Enzyme Characterization

The K_m value of purified POD activity were determined from Lineweaver–Burk reciprocal plots by using different substrates concentrations of H_2O_2, guiacol, *o*-diansidine or phenol and 4-aminoantipyrine [14, 20, 21]. The purified POD activity profile was analyzed using 20 mM guiacol as substrate and 0.05 M acetate; phosphate and Tris HCl buffer in pH range 5.0–9.0 at 37° C. The purified POD activity profile was also analyzed at a temperature ranged from 10–80 °C. The thermal stability was also investigated by measuring the residual activity of the enzyme after 15 min of incubation at different temperatures ranged from 0-80 °C prior to substrate addition. The effect of metal cations was performed by incubating the enzyme for 15 min at 37 °C with 2 mM of cations prior to substrate addition. The percentage of enzyme activity was calculated considering 100% as the highest activity detected in the assay.

3. RESULTS AND DISCUSSION

3.1. Screening of Peroxidase in Cruciferae Family

Although peroxidase have been reported in some *Curciferae* family such as cabbage root [6], turnip root [22], horseradish root [9] and broccoli stem [23], very little information have been reported for production of peroxidase from germinated seeds. In this investigation, peroxidase activity was screened in 6-day germinated seeds for twenty two cultivars of three species of *Cruciferae* family (Table 1). The highest activity level of peroxidase was detected in *Brassica Rapa* cv. Iraqi turnip (specific activity of 461units/mg protein**)**. The low specific activity had been reported for peroxidase from carrot, bean, sweet potato, radish and turnip roots (1.5 to 59 units/mg protein) [24]. The specific activity peroxidase from *Citrus jambhiri* cv. Adalia peel was 751 unit/mg protein [25].

3.2. Purification of Iraqi Turnip Peroxidase

The purification of peroxidase from 6-day germinated seeds of *Brassica Rapa* c.v. Iraqi turnip were summarized in Table 2. The ammonium sulphate fraction contained 4600 units of peroxidase with a specific activity of 836 units/mg protein represented 1.8 fold purification over the crude extract and 66.6% recovery. By Phenyl Sepharose column, four forms of peroxidase (PODI to PODIV) were separated (Fig. 1). This is consistent with peroxidase from sorghum grain [26] which had four isoenzymes. In contrast, two-three isoenzymes from turnip roots [24], two isoenzymes from Chinese cabbage roots [6]**,** three isoenzymes from broccoli stems [23] and six isoenzymes were separated from Korean radish were separated [27].

Completion of purification was carried out for PODII with the highest activity by chromatography on Sephacryl S-200 column (Fig. 2). The purity of Sephacryl S-200 PODII fraction was proved by polyacrylamide gel electrophoresis (Fig. 3a) and its specific activity was 5333 units/mg protein which represented 11.6 fold purification over the crude extract with 25% recovery and RZ value 1.4. Singh

Table 1
Screening of Peroxidase Activity in the Germinated Seeds of three Species of Cruciferae Family

Species	*Sub-Speciesand cultivars*	*Enzyme activity Units/g seeds*	*Protein concentration mg/g germinated seeds*	*Specific activity Units/mg protein*
Brassica	*Brassica Oleracea Var Capitata L*			
	Cabbage F_1 tara	180	0.70	257.1
	Cabbage F_2 tara	70	1	70
	Cabbage F_1 cjn_{10}	52	0.60	86.6
	Cabbage F_1 cjn_{12}	70	0.62	106.3
	Cabbage F_1 red	50	0.55	91.4
	Cabbage F_2 red	100	1.26	97.4
	Cabbage F_3 red	65	1	65
	Cabbage bronswick	116	0.87	132.7
	Cabbage seventy hybrid	26	1.14	22.8
	Cabbage kahera hybrid	22	0.89	24.7
	Cabbage baldi	20	1	20
	Cabbage ganzory	25	0.75	37.5
	Cauliflower	170	1.12	151.7
	Brassica Rapa.L			
	Iraqi turnip Purole top white	250	0.54	461.25
	globe turnip	100	0.75	150
	Soltani turnip	130	1.12	116
	Baladi turnip	70	1	70
Raphanus	*Raphanus Sativum L*			
	Soltani radish	172	1.12	151.4
	Red_1 radish	140	0.45	215.3
	Red $_2$ radis	70	1	70
	Cherry bell radish h	100	1.2	80
Eurca	*Eurca Sativa, mill*	35	0.76	45.9
	Rocket			

Table 2
Purification Scheme of Brassica Rapa c.v. Iraqi Turnip Peroxidase by using Ammonium Sulphate; Phenyl Sepharose and Sephacryl S-200

Step	*Total Activity (Units)*	*Total protein (mg)*	*S.A.(Units/ mg protein)*	*Fold purification*	*Recovery (%)*	*RZ value 405/280*
Crude extract	6900	15	460			0.13
Amm sulphate Precipitation 70 %	4600	5.5	863	1.82	66.6	0.56
Phenyl sepharose CL-4B 1 M Amm. sulphate (POD I)	352	1.8	196	0.426	5.1	0.61
0.8MAmm sulphate (PODII)	2492	1.1	2265	4.9	36.1	0.72
0.6 M Amm. sulphate (PODIII)	498	0.53	940	2.04	7.2	0.61
0.0 M Amm. sulphate (PODIV)	740	0.59	1254	2.725	10.7	0.63
Sephacryl S-200 (POD II)	1760	0.33	5333	11.6	25.5	1.4

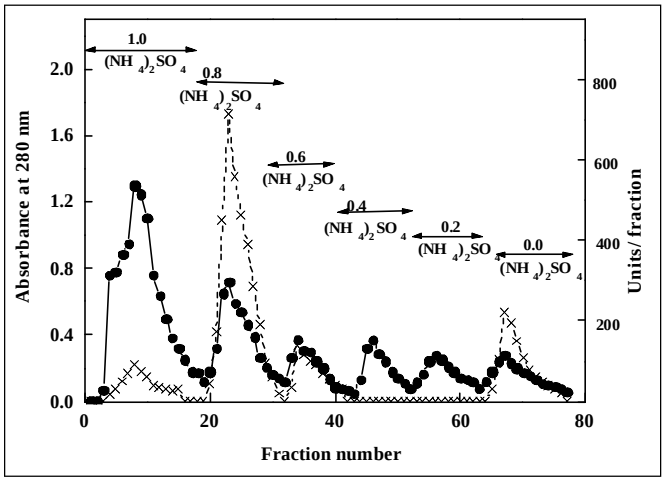


Figure 1: A typical elution profile for the chromatography of Brassica Rapa c.v. Iraqi turnip peroxidase ammonium sulfate precipitation on Phenyl sepharose CL-4B column (6 x 2 cm) previously equilibrated with 50 mM sodium acetate buffer, pH 5.5 containing 1 M ammonium sulphate at a flow rate of 30 ml/h and 3 ml fractions, absorbance at 280 nm (.——.), peroxidase activity (x — —x)

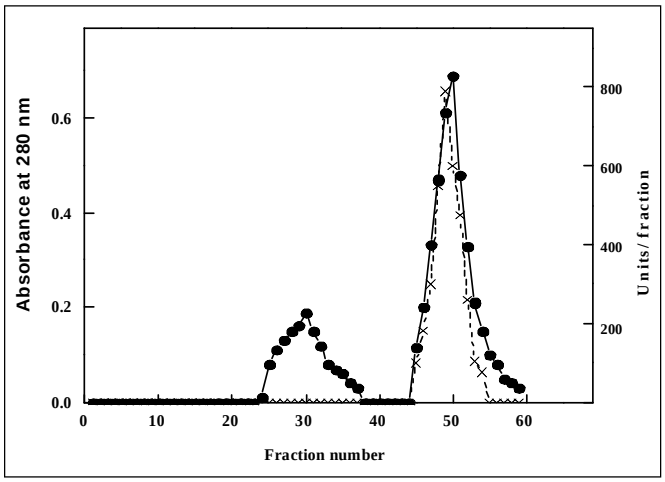


Figure 2: A typical elution profile for Iraqi turnip peroxidase PODII from Phenyl Sepharose fraction on Sephacryl S-200 column (90 x 1.6 cm) previously equilibrated with 50 mM sodium acetate buffer, pH 5.5 at a flow rate of 30 ml/h and 3 ml fractions, absorbance at 280 nm (.———.), peroxidase activity (x —x)

et al. [24] found that turnip peroxidase was purified with a specific activity of 194 units/mg and RZ\ 0.83. Another study showed that purified peroxidase from turnip roots had specific activity 512 units/mg protein with RZ of 3.02 [28].

3.3. Molecular Weight of PODII

The molecular weight of peroxidases from different plant sources ranged from 30 to 60 kDa and the differences observed are attributed to modifications in the polypeptide chain as well as in the number and composition of glycan chains [23]. The molecular weight of PODII was estimated to be 32 kDa. This molecular weight was confirmed by SDS –PAGE as single subunit and estimated to be 32 kDa as single subunit (Fig. 3b). This molecular weight was similar to molecular weight of peroxidases from tomato (34 and 37kDa) [29] and green asparagus (34 kDa) [30] and smaller than those reported from broccoli (48 kDa) [23] and horseradish POD (40-46 kDa) [9]. The majority of peroxidases reported to date are monomers, with the exception of coconut POD which was reported as a tetramer of 55 kDa subunits [31] and palm leaf POD with four subunits of 48kDa [32].

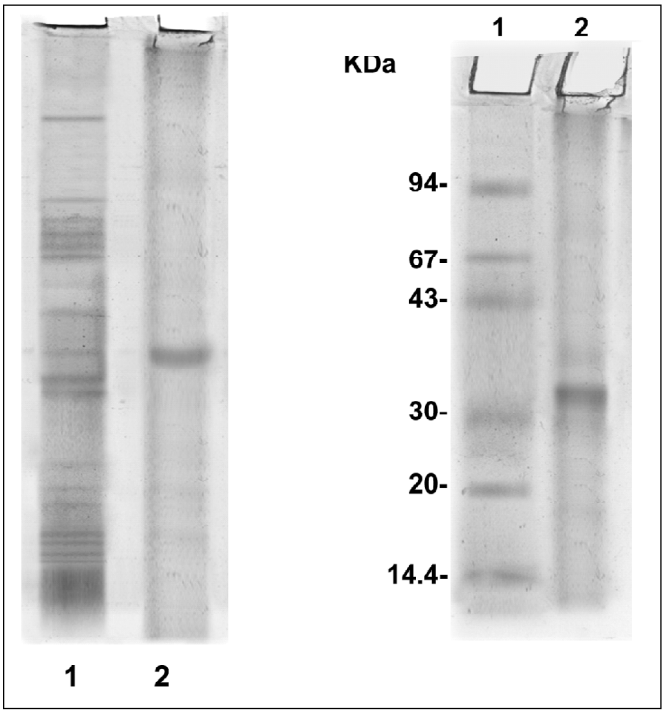


Figure 3: (a) Polyacrylamide gel electrophoresis (PAGE) for Iraqi turnip peroxidase of samples during purification steps; lane 1: crude extract; lane 2: Sephacryl S-200 POD II. (b) SDS-PAGE of the purified POD II; lane1: Standard proteins lane2: Sephacryl S-200 POD II

3.4. Properties of PODII

3.4.1. Substrate Specificity and K_m

Substrate specificity of POD II was studied by using three different chromogenic substrates (guaiacol, 4-aminoantipyrene and *o*-dianisidine). The POD II has more affinity toward guaiacol and the affinity

decreasing in the order of guaiacol > o-dianisidine > 4-aminoantipyrene (Table 3). Therefore, this enzyme could be used for bioremediation of phenolic compounds polluted the environment. Peroxidases reduce hydrogen peroxide and oxidize a wide number of compounds including phenols, aromatic amines and thioanisoles [33].

Table 3
Kinetic Parameters of POD II on Different Substrates

Substrate	K_m *(mM)*	V_{max} *(U/assay)*	$K_{cat} \times 10^3$ (S^{-1})	V_{max}/K_m	K_{cat}/K_m
Guiacol	13.6	2.34	68	0.17	5000
4-Aminoantipyrine	25	1.66	46.5	0.066	1860
o-Dianisidine	16	1.95	56.5	0.1	3530

The K_m value of PODII using H_2O_2 (Fig. 4 a) and three chromogenic substrates (Fig. 3b) were estimated. K_m value of PODII was 2 mM H_2O_2. This value was higher than that reported for peroxidase *from Raphnus sativus* (K_m 1.27 mM) [27], *Jambhiri cv. Adalia* (0.54 mM) [25], and lower than that reported for *Brussels sprouts* (11.4 and 6.2 mM) [34]. K_m values of PODII using chromogenic substrates were 13.6 mM guaiacol, 16 mM *o*-dianisidine and 25 mM 4-aminoantipyrene. Different K_m values were detected for peroxidases from Korean radish roots (13.8 mM guaiacol) [35], *jambhiri cv. Adalia* (11 mM *o*-dianisidine) [25] and olive fruit (41 mM 4-aminoantipyrene) [36].

The kinetic parameters showed also that guaiacol had higher affinity toward PODII than o-dianisidine and 4-aminoantipyrene, where their K_m values were 13.6, 16 and 25 mM, V_{max} were 2.34, 1.95 and 1.66 and V_{max} / K_m were 0.17, 0.1 and 0.06, respectively (Table 3). In *Citrus jambhiri* cv. Adalia, guaiacol and o-dianisidine showed higher affinity toward peroxidase, where their K_m values are 5 and 11 mM, respectively. The values of V_{max} are 18 and 3.12, whereas V_{max}/K_m are 3.6 and 0.28, respectively [25].

3.4.2. pH Optimum

PODII has maximum activity at pH 5.5. A rapid decrease in activity was found on either the basic or acidic sides of the pH optimum (Fig. 5a). An optimum pH of 5.5, 5.0 and 4.0 had been reported for peroxidases isolated from turnip root [22], palm leaf [32] and marula fruit [37], respectively. The

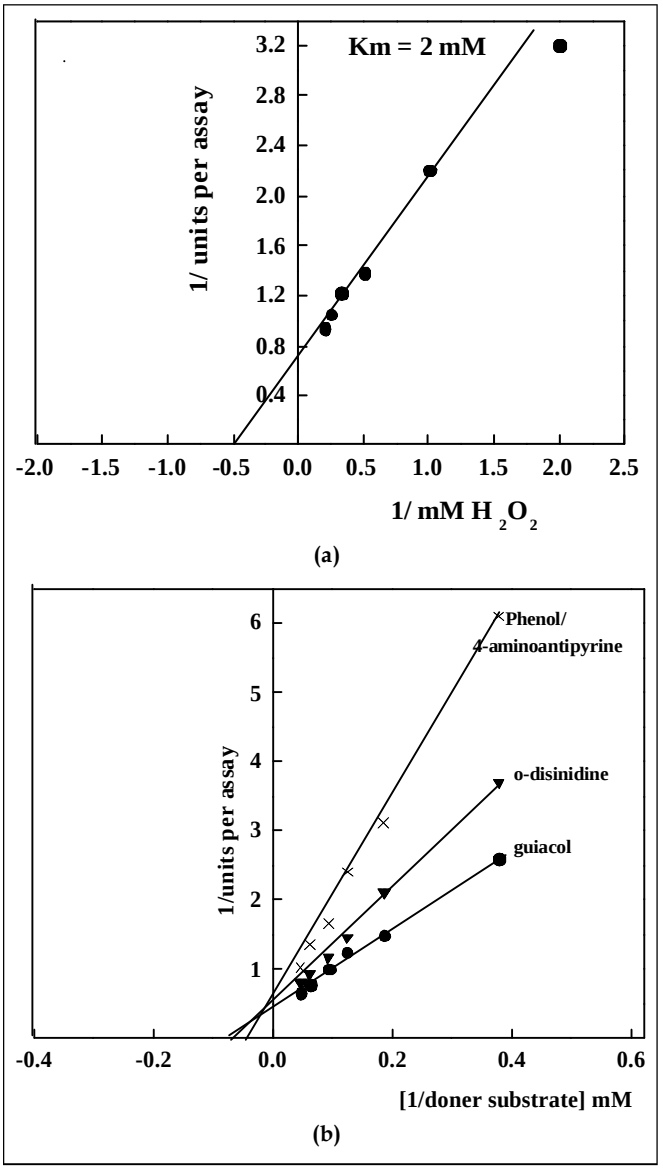


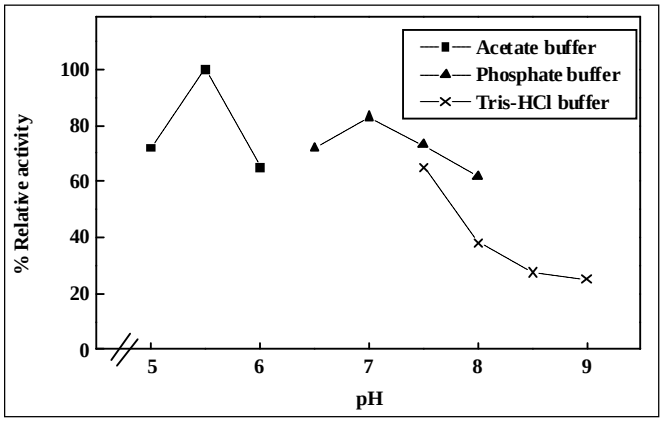
Figure 4: Lineweaver-Burk plots relating PODII reaction velocities to hydrogen peroxide (a) and guaiacol, o-dianisidine, 4-aminoantipyrene (b) substrate concentrations

peroxidase isoenzymes from broccoli were reported to have a pH optimum of 6.0 for neutral enzyme and 4.0 for acidic enzyme [23].

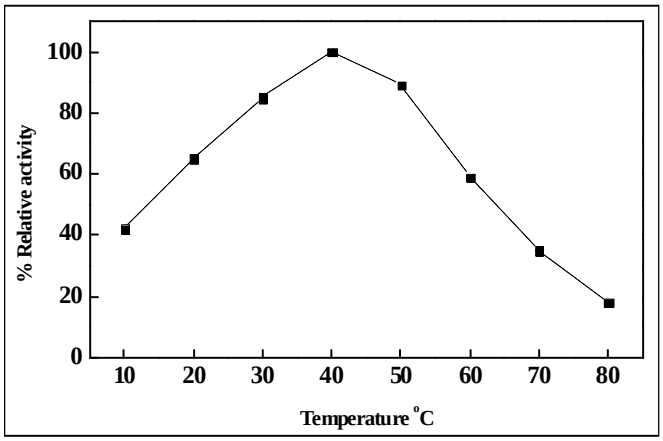
3.4.3. Effect of Temperature

The maximum activity of PODII was detected at 40 °C (Fig. 5b). An optimum temperature in the range of 40 to 55 °C had been reported for turnip peroxidases [22]. Peroxidase from sweet potato tubers has low optimum temperature around 25 °C [38]. POD II is stable up to 30 °C, followed by a decrease in activity till 80 °C (18% remaining

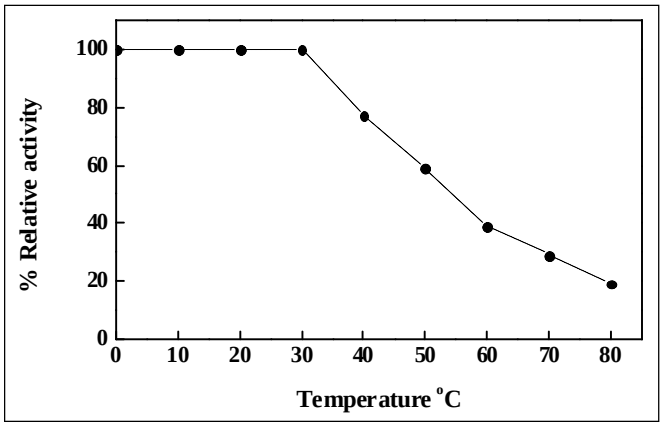
activity) (Fig. 5c). *Citrus jambhiri* cv. Adalia peroxidase is stable at 10-40 °C and unstable above 50 °C [25]. The variability in the heat stability of POD can be attributed largely to the particular enzyme structure.



(a)



(b)



(c)

Figure 5: pH optimum (a), optimum temperature (b) and thermal stability (c) of Iraqi turnip peroxidase (PODII)

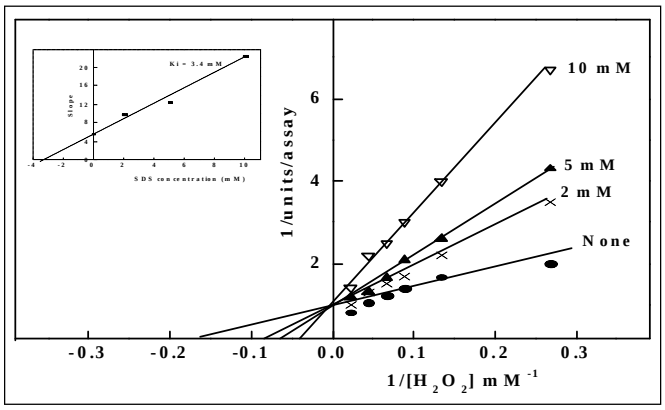
3.4.4. Effect of Metal Cations and Different Inhibitors

The effect of some metal cations at 2 mM on PODII activity was detected (Table 4). The effectiveness of metal cations as inhibitors for PODII was Hg^{2+} >Mn^{2+}>Ni^{2+} > Ba^{2+} >Co^{2+}>Ca^{2+}, Cu^{2+} >Mg^{2+}. Hg^{2+} is potent inhibitor of enzymatic reactions by binding to SH groups present in the active site of enzyme causing its irreversible inactivation [39]. On the contrary, Hg^{2+} is a reversible inhibitor of substrate oxidation by horseradish peroxidase and the type of inhibition depended on the length of incubation of the enzyme with the metal ion and on Hg^{2+} concentration [40]. The correlation between the degree of the inhibition of penut peroxidase by metal ions and their affinity to SH-groups decreased as follows: Hg^{2+} >Pb^{2+} >Cd^{2+} > Zn^{2+} [41].

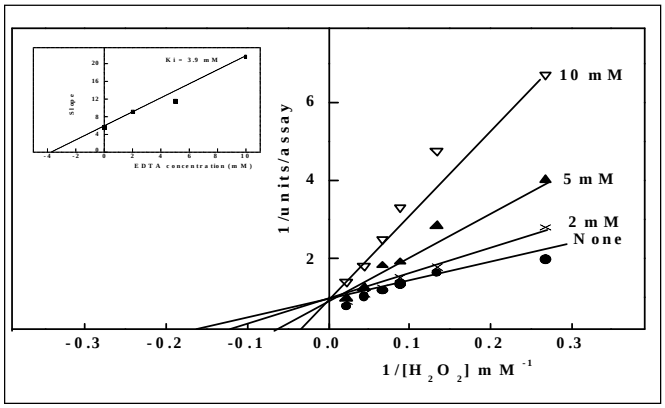
Table 4
Effect of Metal Cations on Activity of Brassica Rapa c.v. Iraqi Turnip Peroxidase (PODII)

Inhhibitor (2 mM)	*% Relative*	*% inhibition activity*
None	100	Zero
Cu^{2+}	77	23
Co^{2+}	75	25
Hg^{2+}	30	70
Ni^{2+}	57	43
Mn^{2+}	41.0	59
Ca^{2+}	77	23
Mg^{2+}	83	17
Ba^{2+}	70	30
SDS	75	25
EDTA	83	17
DTT	70	30

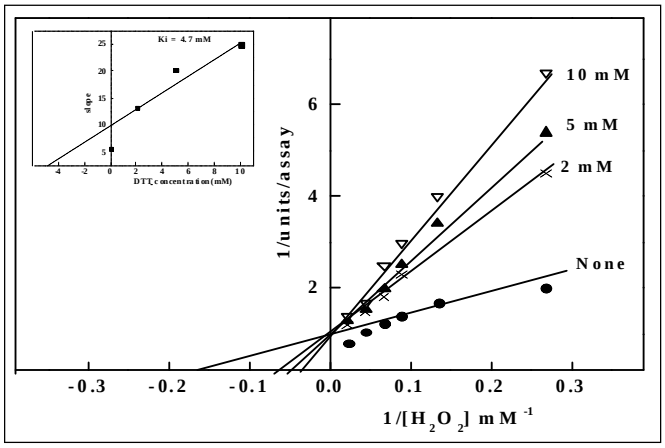
The inhibition effect of sodium dodecyl sulphate, SDS, ethylenediamine tetraacetic acid, EDTA and dithiothretol (DTT) on PODII was examined (Table 4). The inhibition constant (K_i) of SDS, EDTA and DTT were estimated to be 3.4, 3.9 and 4.7 mM, respectively and were found to be competitive inhibitors (Fig. 6). EDTA reacts as a chelating agent of the Fe^{2+} atom found in the peroxidase active center, as well as DTT that inhibits peroxidase activity by reacting with Fe^{2+} in the active center [42]. In white-rot fungus *Schizophyllum* sp. F17, modification of the enzyme with DTT resulted in complete loss of its activity, because DTT can cleave disulfide bond, and peroxidase has five disulfide-bridging elements, which maintain the



(a)



(b)



(c)

Figure 6: Kinetics of inhibition for PODII by SDS (a), EDTA (b) and DTT (c). Reaction mixture contained in 1.0 ml: 0.2 units of enzyme, 50 mM acetate buffer, pH 5.5 and 20 mM guaiacol. H_2O_2 and inhibitor were added in concentrations indicated

structure of the active site [43]. From our results, this enzyme might be potentially used for clinical and industrial applications.

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