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Full Length Research Paper

Purification and characterisation of a plant peroxidase from rocket (*Eruca vesicaria* sbsp. Sativa) (Mill.) (syn. *E. sativa*) and effects of some chemicals on peroxidase activity *in vitro*

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Rocket (*Eruca vesicaria* sbsp. Sativa) (Mill.) (syn. *E. sativa*) was grown and used widely in Turkey as a garnish in salads. A peroxidase (POD) from leaves of rocket (*Eruca vesicaria* sbsp. *Sativa*) was purified using sequential $(NH_4)_2SO_4$ precipitation, CM-Sephadex and Sephacryl S-200 chromatographies. A peroxidase (POD) was purified 220.3-fold from the Rocket (*E. vesicaria* sbsp. *Sativa*) with an overall yield of 80.79%. The purified enzyme has an optimum pH, 6.0 and its optimum temperature was 40°C. The V_{max} and K_M values were determined by Lineweaver-Burk graphics using different substrates. The purification degree and the molecular mass of the enzyme (34 kDa) were determined by SDS-PAGE and gel filtration chromatography. POD enzyme activity was strongly inhibited by Ca^{2+}, Mn^{2+}, Hg_2^{2+}, Zn^{2+} and Fe^{2+} as metal ions and SDS, EDTA, ascorbic acid, dithioeritritol as chemicals. But, Ni^{2+}, Co^{2+}, Cu^{2+} slightly activated the enzyme. They inhibited in the different range of peroxidase activity. Changes of POD enzyme's kinetic parameters were most important during chemicals and metal ions metabolism, because they were risk for environmental pollution.

Key words: Rocket (*Eruca vesicaria* sbsp. Sativa), peroxidase (POD), purification, metal ions.

INTRODUCTION

Peroxidase (POD) (EC 1.11.1.7), an oxidoreductase enzyme catalyzes reactions between compounds which hydrogen atoms tend to give and H_2O_2 as the receiver of atoms. POD catalyzes the oxidation of the organic and inorganic substrates by using hydrogen peroxide. In addition, POD also catalyzes dehydrogenation reaction of a large number of aromatic compounds such as phenols, hydroquinone and hidrokinonid amines (Pütter and Becker, 1987; Van Huytstee, 1987). Peroxidases examined in different groups according to the arrangement of amino acids and structure as animal peroxidases, plant, fungal and bacterial peroxidases. These species of peroxidase is due to the differences of amino acid sequences (Welinder, 1979). In plants, peroxidase enzyme has vital functions such as hormonal regulation, defense mechanisms, lignin biosynthesis and adjustment of the amount of indole acetic acid during the catch up the fruits and vegetables (Welinder, 1979; Wakamatsu and Takahama, 1993; Agostini et al., 1997; Adams, 1978; Duarte-Vazquez et al., 2001). In the contemporary world, environment pollution is one of the most important problems of the living that as a result, it constitute the extreme penetration growth, rapid urbanization and advanced technology, and it is threaten to the natural

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resources. Heavy metal species are some of the most common pollutants that are found in industrial waste-waters. Because of their toxicity, these species can have a serious impact if released into the environment as a result of bioaccumulation and they may be extremely toxic even in trace quantities.

High concentration of heavy metals in the environment can be detrimental to a variety of living species (Mahvi and Bazrafshan, 2007; Nadaroglu and Kalkan, 2012; Nadaroglu et al., 2010). Rocket (*Eruca vesicaria* sbsp. Sativa) (Mill.) (syn. *E. sativa*) is in the family Brassicaceae and it is widely grown in Turkey. In Turkey, rocket is existing as wild in nature as well as are being presently cultivated widely for agricultural purposes. Rocket was often used as garnish Turkey and rocket salad, but it was cooked as food in some countries. This plant is also used as medicine in the stomach disease among the people (Alqasoumi et al., 2009; Lamy et al., 2008). The present investigation reports the isolation, purification and biochemical characterization of peroxidase from rocket leaves. In addition, in the second phase of the research, it was investigated the effects of metal ions, detergent residues and some chemicals on the activity of purified enzyme, because the plant had a very high risk of exposure to these chemicals in the environmental conditions.

MATERIALS AND METHODS

Chemical

Guaiacol, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), pyrogallol, 4-methylcatechol, hydrochinon (1,4-dihydroxybenzol), ethanol, 2-propanol, sodium acetate (CH_3COONa), bovine serum albumin (BSA), CM-sephadex, and sephacryl S-200, ethylenediaminetetraacetic acid (EDTA), dithiothreitol, β-mercaptoethanol and agents for SDS-PAGE were purchased from Sigma (USA). Ammonium sulfate [$(NH_4)_2SO_4$], trichloroacetic acid (TCA, 99%), sodium chloride (NaCl) and potassium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Plant material and storage conditions

Rocket (*Eruca vesicaria* sbsp. *Sativa*) was collected from the Erzurum region of Turkey and was stored at -20°C till further use.

Purification of peroxidase enzyme

All procedure was carried out at 0 to 4°C unless otherwise stated, and the working buffer was 50 mM phosphate buffer (pH 7).

Preparation of crude extract

In the study, leaves of rocket (*Eruca vesicaria* sbsp. *Sativa*) (20 g) were ground in liquid N_2 and then homogenized in a blender with 50 ml of 50 mM KH_2PO_4 (pH: 7) buffer including 0.5% PVP by shaking and centrifuged at 5,000x*g* for 30 min. The homogenates were centrifuged and precipitates were removed. For the purification of the peroxidase enzyme, the following procedure was implemented (Havir and Mchale, 1987; Nadaroglu, 2009).

Ammonium sulfate fractionation

The collapse of $(NH_4)_2SO_4$ was done from 0 to 90% in supernatant with the internals of 0 to 10, 10 to 20, 20 to 30, 30 to 40, 40 to 50, 50 to 60, 60 to 70, 70 to 80 and 80 to 90. Significant activity was not observed below at a range of 0 to 40% $(NH_4)_2SO_4$. The majority of activity was found in the 40 to 60% precipitate. Solid $(NH_4)_2SO_4$ was added to the supernatant to increase the concentration of $(NH_4)_2SO_4$ from 40% of the fraction to 60%. After mixing it in an ice-bath for 1 h with magnetic stirring, it was centrifuged (10,000x*g*, 30 min and 4°C). The supernatant was discarded and the precipitate was dissolved in 0.1 M KH_2PO_4 (pH: 7) buffer and dialyzed against the same buffer (Havir and Mchale, 1987; Nadaroglu, 2009).

Cation-exchange chromatography

The dialyzed suspension after ammonium sulfate precipitation from the aforementioned step was subjected to cation-exchange chromatography on CM-Sephadex fast flow column preequilibrated with 100 mM phosphate buffer, pH 7.0. The column was washed thoroughly with the same buffer until no protein was detected in the eluate. The bound proteins were eluted with the same buffer using a linear gradient of NaCl from 0 to 1 M. Fractions of 3 ml volume were collected at a flow rate of 3 ml/min. Protein elution was monitored spectrophotometrically by measuring the absorbance at 280 nm. Activity was measured by using guaiacol as the assay substrate. The active fractions from each peak were pooled separately from the other peaks and stored at 4°C.

Gel filtration

Active and homogenous fractions from the cation exchange were pooled, desalted and concentrated using Sephadex G25. The resulting enzyme preparation was subjected to gel filtration on a Sephacryl S-200 column (120 × 1 cm) pre-equilibrated with 25 mM phosphate buffer at pH 7 containing 0.5 M NaCl, and the column was eluted isocratically. All the fractions were analyzed as described earlier. The active and homogenous fractions were pooled, concentrated and stored at 4°C for further use (Whitaker, 1963).

Protein concentration

Protein concentration was determined spectrophotometrically (absorbance at 280 nm) as well as by Bradford's method (Bradford, 1976), using bovine serum albumin (BSA) as the standard.

Determination of peroxidase enzyme activity

Peroxidase (POD) activity was carried out spectrophotometrically using guaiacol/H_2O_2 as substrate (Lobarzewski et al., 1990). The increase in the absorption as a result of the formation of the oxidized product (tetraguaiacol) was measured at 470 nm. Reaction mixture contained 100 mM phosphate buffer (pH 6.0), 5 mM guaiacol, and 0.5 mM H_2O_2 at 25°C. The changes in absorbance were read for 3 min using a UV-vis spectrophotometer (T80 UV-VIS Spectrophotometre). Substrate specificity and classification of rocket peroxidase enzyme was determined using different substrates with similar reaction mixture and assay conditions. All the substrates and H_2O_2 were at a fixed concentration of 0.5 mM.

The rate of oxidation of guaiacol, was followed at 470 nm, (ε_{470} = 26.6 mM^{-1} cm^{-1}), ABTS at 734 nm (ε_{734} = 1.5 × 10^4 $M^{-1}cm^{-1}$), pyrogallol at 430 nm (ε_{430} = 2.47 mM^{-1} cm^{-1}), 4-methylcatechol at 412 nm, (ε470 = 1010 M^{-1} cm^{-1}) and hydroquinone (1.03 mM^{-1} cm^{-1}). One unit of enzymatic activity was defined as the amount of enzyme that oxidizes 1 µmol/min of hydrogen donors under assay conditions.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme. It was carried out in 3 and 10% acrylamide concentrations for the stacking and running gels, respectively, each of them containing 0.1% SDS (Laemmli, 1970). The sample (20 µg) was applied to the electrophoresis medium. Brome tymol blue was used as tracking dye. Gels were stained in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol, 10% acetic acid and 40% distilled water for 1.5 h. It was destained by washing with 50% methanol, 10% acetic acid and 40% distilled water several times (Laemmli, 1970). The electrophoretic pattern was photographed (Figure 3).

Molecular weight determination by gel filtration

A column (3 × 70 cm) of Sephadex G100 was prepared. The column was equilibrated with the buffer (0.05 M Na_2HPO_4, 1 mM dithioerythritol, pH: 7) until the absorbance was zero at 280 nm. The standard protein solution (bovine serum albumin, 66 kDa; egg ovalbumin, 45 kDa; pepsin, 34 kDa; trypsinogen, 24 kDa; β-lactoglobulin and lysozyme, 14 kDa) was added to the column. The purified peroxidase enzyme was added into the column separately and then eluted under the same conditions. The flow rate through the column was 20 ml/h. The elution volume was compared with standard proteins (Whitaker, 1963).

Dependence of enzyme activity on pH and temperature

The effect of pH on the enzymatic activity of the purified enzyme was determined within the range of pH 2.0 to 11.0. The buffers used were glycine-HCl (pH 2.0 to 3.0), sodium acetate (pH 4.0 to 5.5), sodium phosphate (pH 6.0 to 7.5), Tris (pH 8.0 to 10.0) and glycine (pH 10.5 to 12.0). Activity measurements were separatelly conducted as described earlier by using guaiacol, ABTS, pyrogallol, 4-methylcatechol and hydrochinon as a substrate. Similarly, an analysis of the effect of temperature on the enzyme activity was conducted to determine the optimum temperature. Enzyme samples were incubated at different temperatures in the range of 20 to 90°C for 15 min, and an aliquot was used for activity measurement at the same temperature for substrates of guaiacol, ABTS, pyrogallol, 4-methylcatechol and hydrochinon, separately.

Effect of pH and temperature on the peroxidase stability

The ability of the peroxidase to retain its activity under conditions of varying pH (2 to 11) and temperature (40 to 80°C) was investigated. The enzyme was incubated under specified conditions of pH for 24 h, and the residual activity was determined. In the case of temperature stability measurements, the sample was incubated at the desired temperature for 15 min and the residual activity was measured as described earlier. Kinetic parameters V_{max} and K_M values were separately determined with ABTS/H_2O_2, guaiacol/H_2O_2, ABTS/H_2O_2, pyrogallol/H_2O_2, 4-methylcatechol/H_2O_2 and hydrochinon/H_2O_2 as a substrate. Kinetic parameters were determined for each substrates using the Lineweaver–Burk double reciprocal plot.

Effect of various inhibitors on the peroxidase activity

Effect of some metal ions on the peroxidase activity

The effect of various metal ions (Ca^{2+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+} and K^{+}) on purified peroxidase were investigated. Each inhibitor solution was prepared at six different concentrations (0.084 to 1.67 mM), and each solution was added in a cuvette containing 0.5 m enzyme. Its total volume was adjusted to 3 ml with buffer solution. A control assay of the enzyme activity was done without inhibitors and resulting activity was taken as 100%. The effect of each agent was determined by measuring the enzyme activity using the guaiacol as a substrate.

Effect of some compounds on the peroxidase activity

The effect of various compounds on the activity of purified peroxidase enzyme was determined using thiol specific inhibitors, activators, non-specific compounds and detergent. The used compounds were sodium dodecyl sulfate (SDS), ethylene-diaminetetraacetic acid (EDTA), ascorbic acid, dithioerythritol, 5 mM. The effects of these compounds on the activity of purified peroxidase enzyme were performed as described earlier.

Statistical analysis

All of the tests were conducted in triplicate for determination of the peroxidase activities of samples. Data were expressed as means ± standard errors. Statistical analyses were performed using SPSS version 10.0 software (SPSS Inc., Chicago, IL, USA), and the significant differences were determined with a 95% confidence interval ($p < 0.001$ and 0.05) using Tukey's test.

RESULTS AND DISCUSSION

A new plant peroxidase from the leaves of rocket *(Eruca vesicaria* sbsp. Sativa*)* was purified and characterized by precipitating in $(NH_4)_2SO_4$ followed by cation-exchange and gel filtration chromatograph. Guaiacol was used as a substrate in the determination of activity in the protein eluted from the CM-Sephadex column and Sephacryl S200 column. The results pertaining to purification of peroxidase using all purification techniques are summarized in Table 1. Ammonium sulfate $(NH_4)_2SO_4$ fractionation, an extensively used technique in enzyme purification was performed as a first purification step. The enzyme obtained from the crude extract of the rocket (*Eruca vesicaria* sbsp. Sativa), using 40 to 60% $(NH_4)_2SO_4$ saturation with 27.96-fold purification and 33.45% recovery, was subjected to ion-exchange chromatography, which gave one peak with peroxidase activity. Passage from CM-sephadex column further purified the enzyme to 100.5-fold with a recovery of 62.2% and speciWc activity of 6.85 EU/mg. Then, only one peak with peroxidase activity was obtained when the partially purified enzyme was applied to Sephacryl S 200

Table 1. Purification scheme for rocket peroxidase.

Fractions	Volume (ml)	Activity (EU/ml)	Total activity		Protein (mg/ml)	Spesific activity (EU/mg protein)	Purification
			EU	%			Fold
Homogenate	50	18.13	5906.5±0.12	-	8.53±0.11	2.13	-
$(NH_4)_2SO_4$ precipitetion (40-60%)	20	8.55	1651.0±1.21	27.96	0.12±0.65	71.25	33.45
CM-Sephadex ıon exchange chromatography	15	6.85	1026.75±0.21	62.19	0.032±0.45	214.06	100.5
Sephacryl S 200 gel filtration chromatography	15	5.63	829.5±0.78	80.79	0.012±1.65	469.17	220.27

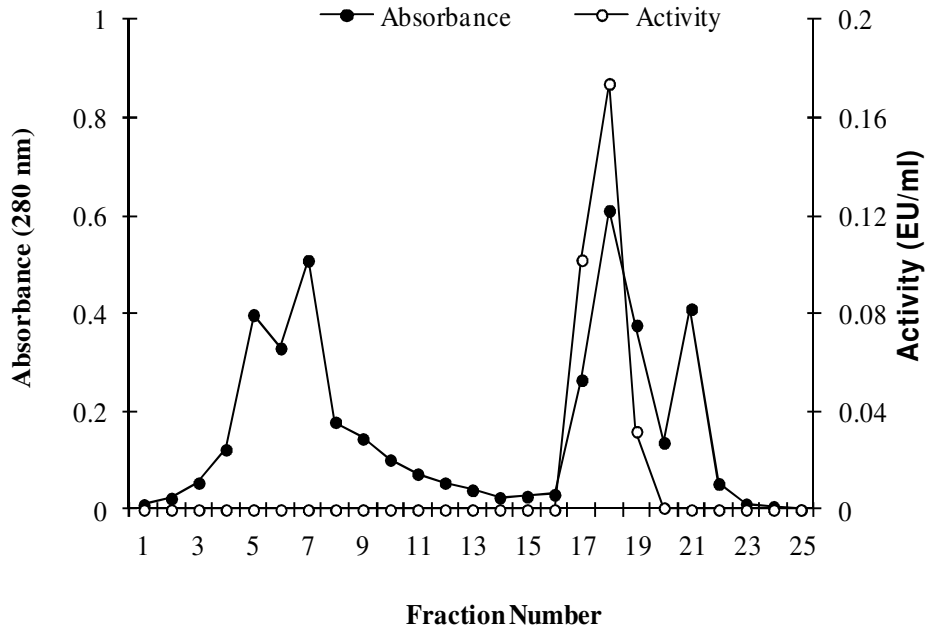

Figure 1. Typical elution profile for the chromatography of rocket peroxidase on CM-Sephadex column.

column.

An overall 220.3-fold purity was achieved with a yield of 80.8% and specific activity of 5.63 EU/mg. The final purification of 220.3-fold suggested that the peroxidase is highly abundant in the rocket (*Eruca vesicaria* sbsp. Sativa). The elution profiles consisting of a peroxidase, which was purified from the rocket (*E. vesicaria* sbsp. Sativa) using cation-exchange chromatography and gel filtration chromatography, was shown in Figures 1 and 2.

The purified peroxidase was examined by SDS electrophoresis (Figure 3). As shown in Figure 3,

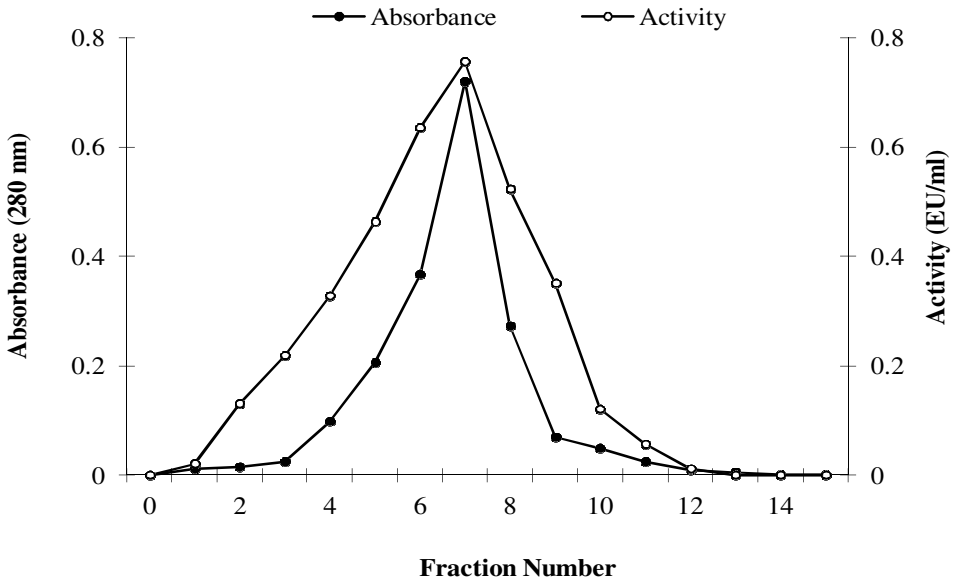

Figure 2. Gel filtration of rocket peroxidase on Sephacryl S-200 column.

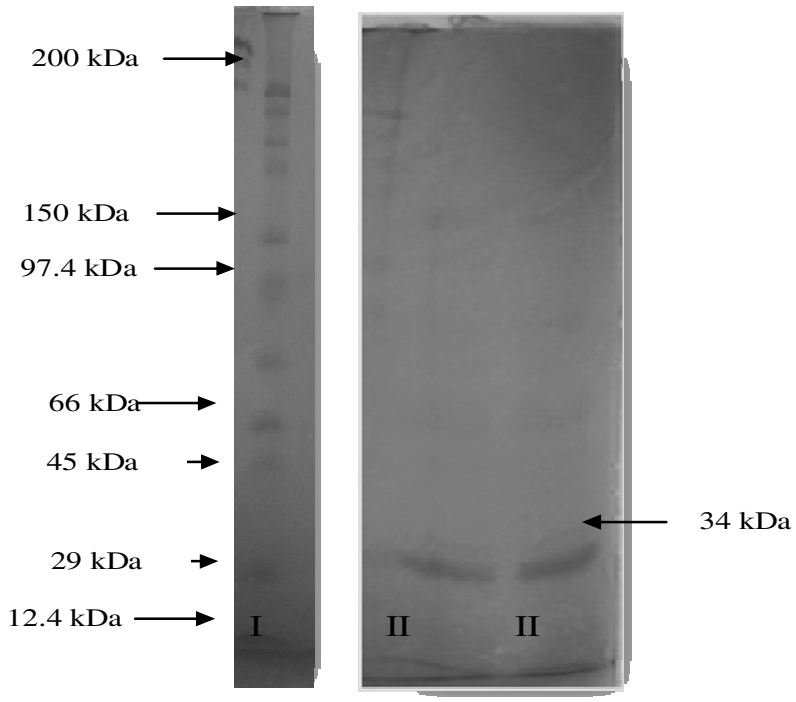

Figure 3. SDS-PAGE electrophoretic pattern of peroxidase [standart protein (β-Amylase, sweet potato, 200 kDa; alcohol dehydrogenase, yeast, 150 kDa bovine serum albumin, 97.4 kDa; rabbit muscle phosphorylase A, 66 kDa; egg ovalbumin, 45 kDa; pepsin, 29 kDa; carbonic anhydrase); cytochrome c, horse heart 12.4 kDa (I); purified laccase enzyme from rocket peroxidase (II)].

SDS-PAGE revealed a single protein band. The molecular weight of the enzyme was determined as 34 kDa by using the gel filtration chromatograph and compared with known standard proteins. The molecular weight of the purified peroxidase was determined by using the following protein standards: bovine albumin (66

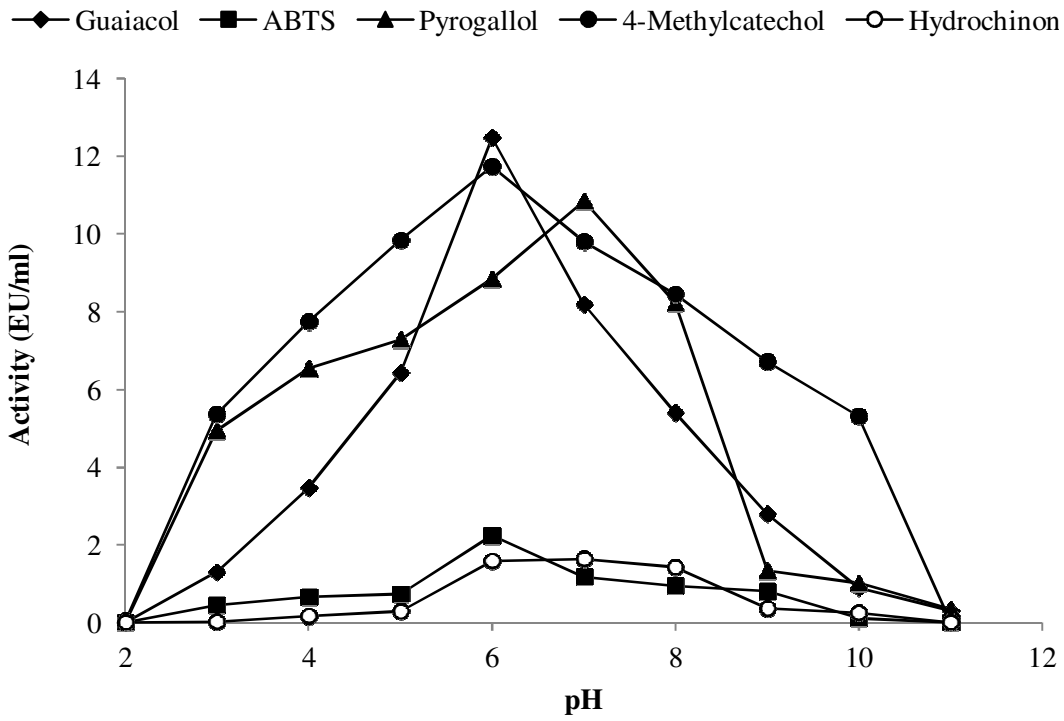

Figure 4. Effect of pH on the activity of the purified peroxidase. Enzymes and substrate were dissolved either in 10 mM buffers of various pH. Other conditions were as given for the standard assay method.

kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa).

The molecular weight for most plant peroxidases has within the range 40 to 50 kDa. It is indicated that they are a slightly wider range of 20 to 54 kDa for peroxidases from oranges (22 to 44 kDa, peanuts (40 to 42 kDa), horseradish (44 kDa), *Raphanus sativus* (44 kDa), sweet potato (37 kDa) and olive fruit (18 to 20 kDa) (Clemente, 1998; Hu et al., 1989; Kim and Lee, 2005; Leon et al., 2002; Welinder, 1992; Saraiva et al., 2007; Robinson, 1991). These plant peroxidases are similar with the present study.

Effect of pH on rocket peroxidase activity

Optimum enzyme activity was observed at pH 6.0 for substrates of guaiacol and ABTS, at 7 for 3-methylcathecol and 7.5 for hydrochinon as shown in Figure 4. This broad range of acidic pH dependence for activity made this enzyme interesting for industrial applications especially in the food industry and it also suggest that this enzyme has better function under acid conditions (Tipawan and Barrett, 2005). This pH optimum of the rocket peroxidase closer to palm (*Roystonea regia*) (Sakharow et al., 2002), clover peroxidase (Criquet et al., 2001) and *Prangos ferulacea* (Apiaceae) (Nadaroglu, 2009) by using pyrogallol, guaiacol, ABTS as a substrate.

Effect of temperature of the purified peroxidase

By using guaiacol/H_2O_2, ABTS/H_2O_2, pyrogallol/H_2O_2, 4-methylcatechol/H_2O_2 and hydrochinon/H_2O_2 as subtrate, the optimal temperature for enzyme activity was found to be 40°C, and about 35.0 and 19.2% of the optimum activity was still detectable at 70 and 80°C, as shown in Figure 5. The peroxidase was stable at 40°C and lost 18 and 19% of its activity after 50 and 60 min at 40°C, respectively (Figure 6). This purified peroxidase has high thermal stability. So it indicates that it is an excellent enzyme for the pharmaceutical, food and detergent industries. Most plant peroxidases show optimum activity in the temperature range of 30 to 45°C (Criquet et al., 2001).

Determination of V_{max} and K_M values

V_{max} and K_m values were determined by using different substrates (guaiacol/H_2O_2, ABTS/H_2O_2, pyrogallol/H_2O_2, 4-methylcatechol/H_2O_2 and hydrochinon/H_2O_2) at optimum pH: 6 and 40°C by means of the Linewaver-Burk graph. K_m and V_{max} values for five different substrates are shown in Table 2. The peroxidase exhibited the greatest activity with ABTS (2631 U/mg).

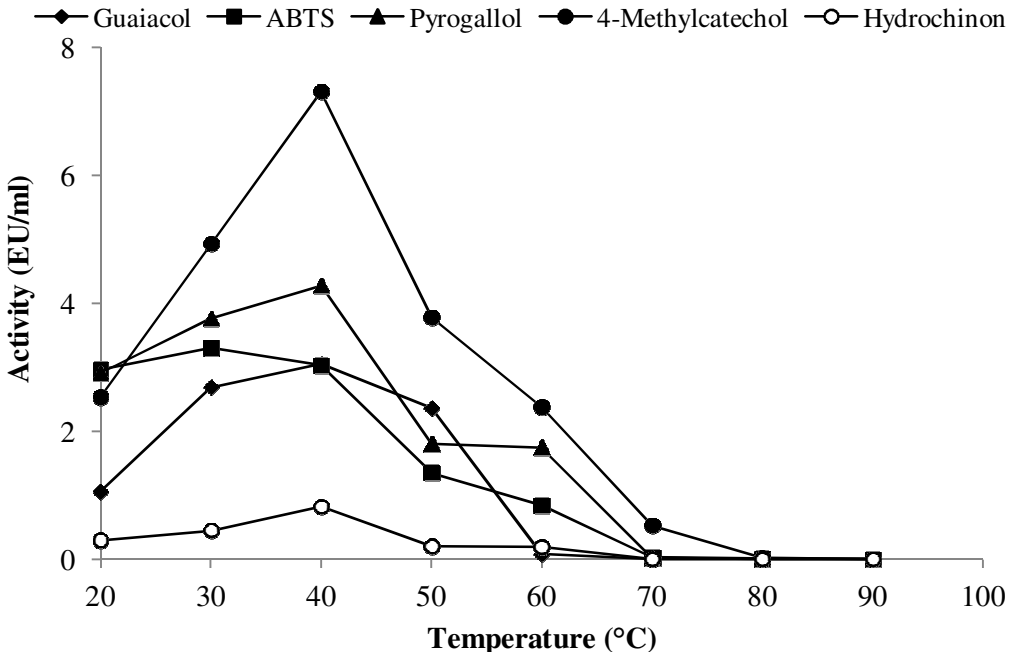

Figure 5. Effect of temperatures on the activity of purified peroxidase. Activity was determined at different temperatures and at pH 6.0 over 10 min using the standard assay method.

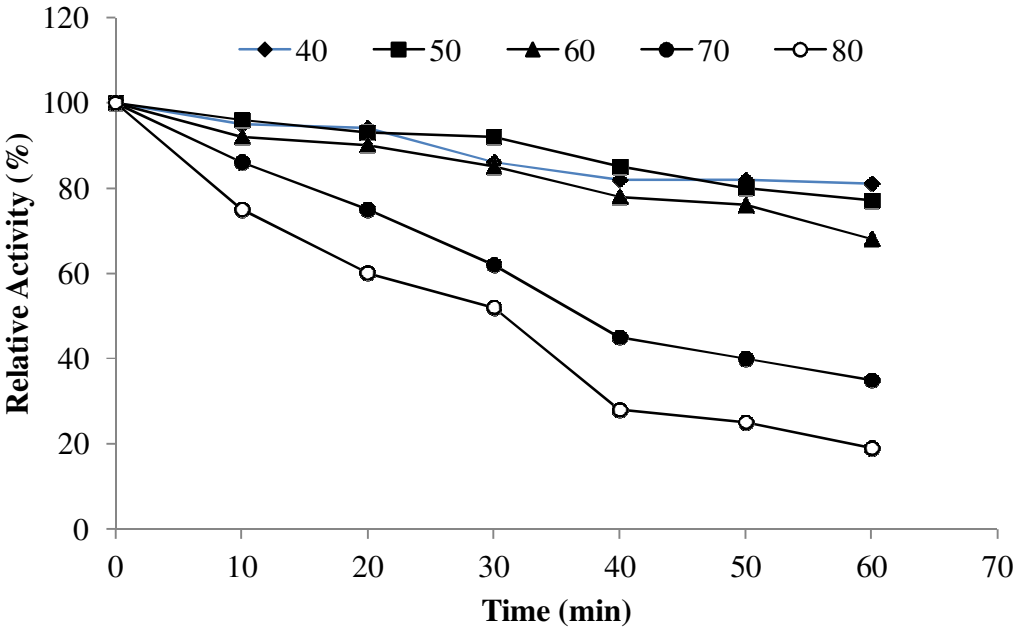

Figure 6. Effect of the temperature on the stability of the purified peroxidase. Enzymes were incubated at pH:6 for 1 h at different different temperature, and the residual activity was measured the standard assay method.

Effect of inhibitors on activity of rocket peroxidase

Although, several metal cations had partial inhibitory effects toward rocket peroxidase, Ni^{2+}, Co^{2+}, Cu^{2+} and Ba^{2+} activated the activity 193, 205, 135 and 106% at 1.67 mM, respectively (Table 3). Mn^{2+}, Hg_2^{2+}, Zn^{2+} and K^{+} had inhibited the rocket peroxidase activity at about 50% at 1.67 mM. The enzyme retained less than 40% of its

Table 2. Determination of K_M and V_{max} for different substrates.

Substrat	K_M (mM)	V_{max} (µmol/L.dak)
Guaikol	375.74	0.314
ABTS	2631.56	5.376
Pyrogallol	510.14	0.89
4-Methyl cathecol	1535.65	3.65
Hidrokinon	65.19	0.303

Table 3. Effect of metal cations on rocket peroxidase.

Concentration (mM)	$CaCl_2$	$BaCl_2$	$MnCl_2$	$NiCl_2$	$CoCl_2$
	Relative activity (%)				
0	100	100	100	100	100
0.084	62.53	53.18	92.15	143.80	152.66
0.167	68.85	54.18	74.18	150.63	172.15
0.5	58.48	67.85	73.92	162.0	179.24
0.84	47.1	60.76	58.73	173.16	186.08
1.167	37.2	83.80	56.23	184.8	192.91
1.67	36.2	105.57	55.699	193.42	205.06

Concentration (mM)	Hg_2Cl_2	$ZnCl_2$	$FeCl_2$	KCl	$CuCl_2$
	Relative activity (%)				
0	100	100	100	100	100
0.084	80.5	76.1	73.58	99.16	156.95
0.167	76.1	74.2	46.16	93.44	155.44
0.5	73.5	69.06	42.58	88.69	136.95
0.84	71.7	68.18	36.98	86.31	136.68
1.167	71.38	63.08	35.22	83.21	135.95
1.67	69.81	60.12	32.33	80.0	135.19

Table 4. Effect of some chemicals on rocket peroxidase.

Concentration (mM)	SDS	EDTA	β-mercaptoethanol	Ditihierithritol
	Relative activity (%)			
0	100	100	100	100
0.084	56.35	109.64	32.15	9.37
0.167	43.52	95.53	23.22	2.77
0.5	11.45	12.15	10.05	0.66
0.84	6.42	8.9	2.12	0.28
1.167	4.09	7.68	0	0
1.67	2.58	3.2	0	0

activity in the presence of 1.67 mM Ca^{2+} and Fe^{2+}. In addition, SDS, EDTA, β-mercaptoethanol and dithioeritritol caused strongly inhibitory effects toward rocket peroxidase activity (Table 4). Inhibition by EDTA was 3.2%; this indicated that the enzyme has metal ion as cofactor. The effect of metal ions on peroxidase activity has been largely described previously. Similar results have been observed with peroxidase of plants (Wong, 1995; Ajila and Rao, 2009; Marquez et al., 2008; Onsa et al., 2004).

Conclusion

This study describes the purification and enzymatic properties of an acidic peroxidase from the rocket (*E.vesicaria* sbsp. Sativa). The results indicate that purified peroxidase wide substrate specificity and stability over a wide range of pH, temperature, metal ions and some chemicals. Therefore, rocket leaves are a potential source of peroxidase for bioanalytical or biotechnological applications, such as enzymatic reagents for clinical diagnosis, food analysis, biotransformation and degredation of various chemicals.

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ABBREVİATİONS

POD, Peroxidase; **PAGE,** polyacrylamide gel electrophoresis; **SDS,** sodium dodecyl sulfate; **EDTA,** ethylenediaminetetraacetic acid; **ABTS,** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); **$(NH_4)_2SO_4$,** ammonium sulfate; **TCA,** trichloroacetic acid.

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