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PEROXIDASES OF SAUDI DATE CULTIVARS (*PHOENIX DACTYLIFERA* L.) IN TAMER STAGE

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Abstract:-Peroxidase activities were screened in sixteen cultivars of Saudi dates in tamer stage. The date cultivars with highest peroxidise activity accompanied with enhancementof the intensity of the browning which was essential character in tamer.Cationic peroxidase I from Barni was purified using ammonium sulphate, ionexchange chromatography and gel filtration. Molecular weight of peroxidase I was 64 kDa. o-Phenylenediaminewas the best substrate for the enzyme compared to other substrates. The Km values of peroxidase I for H2O2, o-phenylenediamine, guaiacol and o-dianisidine were 2.17, 5, 5.55 and 20 mM/ml, respectively. Peroxidase I was found to have temperature optimum at 40℃ and the enzyme activity was remained stable up to 40℃. Peroxidase I exhibited broad pH optimum from 5.5 to 6. At 1 mM concentration, Fe3+ was able to greatly enhance the activity of peroxidase I by 334%. In conclusion, the characterization of peroxidase I could be useful in dates processing.

Keywords:dates; Phoenix dactylifera;cultivars; peroxidase, browning.

INTRODUCTION

Saudi Arabia represents the third world producer of date fruits (Eleid, 2008). In the last decade, Saudi Arabia paid more attention for date palm cultivation. It was clear that the dates production had a minimum level about 527 thousand ton in 1990 and a maximum level by about one million ton in 2010 (Elsabea, 2012). Saudi Arabia had many varieties of dates. The varieties of dates changed according to the different environmental situation of production areas. Dates were considered as the most commonly consumed fruits in Saudi Arabia (Elsabea, 2012). Dates were rich in carbohydrates, poor in fats and proteins, and good source of minerals such as calcium, iron, magnesium, phosphorus, potassium and zinc (Kchaoua*et al.*, 2013; Biglari*et al.*, 2008). In addition, dates were rich in biologically active compounds especially antioxidants, which quenched reactive oxygen species and prevent the oxidation of proteins and lipids (Al-Najadaand Mohamed, 2014; Atmaniet al., 2009).

Browning of fruits and vegetables during post-harvest handling and processing was one of the main causes of quality loss (Mathew andParpia, 1971). This phenomenon was mediated by endogenous enzymatic activities such as polyphenol oxidase and peroxidase(Lee, 1992). The main function of peroxidaseswas the oxidation of phenolic compoundsin presence of H_2O_2(Robinson, 1991). Peroxidases involved in the wall-buildingprocesses such as oxidation of phenols, suberization,and lignification of host plant cells during the defense reactionagainst pathogenic agents (Chittoor*et al.*,1999; Kolattukudy*et al.*, 1992). The involvement of peroxidases in enzymatic browning has been assumed by numerousauthors (Al-Najada and Mohamed, 2014, Dogan*et al.*, 2007). On the other hand, peroxidase could contribute to deteriorating changes in flavor, texture, color, and nutrition in improperly processed fruits and vegetables (Robinson, 1991). The formation of off-flavors in canned fruits and vegetables had been associated with residual peroxidase activity following processing (Lu and Whitaker, 1974). In orange, the level of peroxidase in the juice was associated with loss of flavor quality (Bruemmeret al., 1976). In addition, the involvement of peroxidase as an active oxygen-detoxifying enzyme on postharvest rind staining occurring in citrus fruit in a non-chilling temperature had been investigated (Sala andLafuente, 2004).

Dates could be consumed at three stages of their development mainly bisri, rutab and tamer depending on

cultivars characteristics especially soluble tannin levels, climatological conditions and market demand (Awad, 2007). On the contrary of many fruits, the enzymatic browning by peroxidase may be essential to character of dates in tamer stage (Al-Najada and Mohamed, 2014). Therefore, we screened the peroxidase activity in sixteencommercial cultivars of dates in tamer stage. Peroxidase from cultivarBarni, with the highestactivity, has been purified to homogeneity, and its kinetics for different phenolic compounds and pH and temperature optima had been studied.

MATERIALS AND METHODS

Plant material:

Sixteen cultivars from Saudi dates palm fruits *Phoenix dactylifera* (Agwah, Loban, Mabroum, Khudari, Safawi, Sefrei, Nabtat Ali, Red Sukarrei, Shalabe, Saqeei, Sukarrei, Khlase, Roshodi, Dahlah, Barni and Anberi) were harvested fromAl-madinah Al-munawwarah, Kingdom of Saudi Arabia. All cultivars were obtained in tamer stage and stored at 4 oC until use.

Preparation of crude extract:

Two g from Saudi date palm peel mixed with 10 ml of 20 mMTris-HCl buffer, pH 7.2 and homogenized using mortar. The homogenate was subjected to refrigerated centrifuge at 12,000 rpm for 10 min at 4oC. The supernatant was designated as crude extract.This preparation was repeated three times for each cultivar.

Purification of peroxidase form dates palm cv. Barni

Ammonium sulphate precipitation:

The crude extract of peroxidase from cv. Barni was precipitated by solid ammonium sulphate up to 80% saturation. The precipitate was collected by refrigerated centrifuge at 12,000 rpm for 20 min and dissolved in a least volume of 50 mMTris-HCl buffer, pH 7.2 and dialyzed against the same buffer overnight. The dialysate was centrifuged at 15,000 rpm for 20 min. The supernatant was kept at -15 oC for further purification steps.

Chromatography on DEAE-Sepharose:

The supernatant was loaded on a DEAE-Sepharose CL-6B column (10 x 1.6 cm i.d.) previously equilibrated with 20 mMTris-HCl buffer, pH 7.2. The enzyme was eluted with a stepwise gradient from 0.0 to 0.3 M NaCl in the same buffer. Fractions in 3 ml volume were collected at a flow rate of 60 ml/h. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. Protein fractions exhibiting peroxidase activity were pooled in two peaks (peroxidases I and II).

Sephacryl S-200 column:

Peroxidase I containing the highest activity was concentrated through dialysis against solid sucrose and loaded on Sephacryl S-200 column (90 x 1.6 cm i.d.) previously equilibrated with 20 mMTris-HCl buffer, pH 7.2 and developed at a flow rate of 30 ml/h and 3 ml fractions were collected.

Protein determination:

Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Peroxidase assay:

Peroxidase activity was carried out according to Yuan and Jiang (2003). The reaction mixture (1 ml) contained 8 mM H_2O_2, 40mMguaiacol, 50 mM sodium acetate buffer, pH 5.5 and 0.1 unit of enzyme. The change of absorbance at470 nm due to guaiacol oxidation was followed at 30 s intervals.One unit of peroxidase activity was defined as the amount of enzymewhich increased the optical density (OD) by 1.0 U/min under standardassay conditions.

Molecular weight determination:

Molecular weight was determined by gel filtration, using Sephacryl S-200. The column (90 x 1.6 cm i.d.)

was calibrated with cytochrome C (12.4kDa), carbonic anhydrase (29kDa), bovine serum albumin (67kDa), alcohol dehydrogenase (150kDa) and β-amylase (200kDa). Dextran blue (2000kDa) was used to determine the void volume (V0). Subunit molecular weight was estimated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). SDS denatured phosphorylase b (94kDa), bovine serum albumin (67kDa), ovalbumin (43kDa), carbonic anhydrase (30kDa), soybean trypsin inhibitor (20kDa) and a-lactalbumin (14.2 kDa) were used for the calibration curve.

Characterization of peroxidase

Substrate specificity:

The enzyme was tested to determination a preference for different substrates such as guiacol, o-phenylenediamine, o-dianisidine, pyrogallol and catechol. The activity with guiacol was taken as 100% and % relative activity was determined against different substrates.

Km:

Michaelis–Menten constants were determined by measuring the initial rates of phenolic compound oxidation at 25 °C at different H_2O_2 (2-18 mM), guaiacol (10-80 mM), o-phenylenediamine (5-40 mM) and o-dianisidine (10–50 mM) concentrations. The apparent Km values were determined from Lineweaver–Burk plots at optimum pH and temperature conditions.

Optimum temperature:

Peroxidase activity was determined at a temperature range of 30-70^{o}C. The maximum activity was taken as 100% and % relative activity were plotted against different temperatures.

Thermal stability:

The enzyme was incubated at a temperature range of 30-70^{o}C for 15 min prior to substrate addition. The % relative activity was plotted against different temperatures.

Optimum pH:

Peroxidase activity was determined at various pH using different buffers, sodium acetate (pH 4.0–6.0) and Tris–HCl (pH 6.5–8.5) at 50 mM concentration. The maximum activity was taken as 100% and % relative activity plotted against different pH values.

Effect of metal ions:

The enzyme was incubated with 2 or 5 mM solution of Fe^{3+}, Fe^{2+}, Co^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and $Hg^{2+}$for 15 min prior to substrate addition. The enzyme activity without metal ions was taken as 100% and % relative activity was determined in the presence of metal ions.

Effect of metal chelators:

Peroxidase activity was determined in the presence of metal chelators, EDTA, sodium citrate, sodium oxalate and 1,10-phenanthroline at a concentration of 2 or 5 mM. The enzyme activity without metal chelators was taken as 100% and % relative activity was determined in the presence of metal chelators.

Browning color determination:

The brown color of date crude extract was measured at 420 nmaccording to Wrolstad (1976). Higher values of absorbance at420 nm correspond for higher browning of the tissue.

Statistical analysis:

The obtained data were statistically analyzed as a randomized complete block design with three replicates by analysis of variance (ANOVA) using the statistical package software SAS (SAS Institute Inc., 2000, Cary, NC.,

USA). Comparisons between means were made by F-test and the least significant differences (LSD) at level P = 0.05. Correlations coefficient among the different parameters were also calculated by SAS.

RESULTS AND DISCUSSION

The quantitative screening of peroxidase activities were detected in peel of sixteen cultivars from Saudi dates palm *Phoenix dactylifera* (Agwah, Loban, Mabroum, Khudari, Safawi, Sefrei, Nabtat Ali, Red Sukarrei, Shalabe, Saqeei, Sukarrei, Khlase, Roshodi, Dahlah, Barni and Anberi) (Fig. 1). According to the results of peroxidase activity three different groups could be distinguished. The cultivars with a relatively high peroxidise activity (49.7-102.7 U/g f.w.) were Barni, Agwah, Safawi, Khlase, Red Sukarrei and Sukarrei, respectively. In this respect, Barni cultivar showed the highest peroxidise activity compared with all other cultivars. The cultivars that showed a relatively moderate peroxidise activity (24.8- 38.3 U/g fw) were Shalabe, Roshodi, Sefrei, Nabtat Ali and Mabroum, respectively. While the cultivars Khudari, Saqee, Anberi, Dahlah and Loban showed the lowest peroxidise activity (1.4-13.5 U/g fw), respectively. In this respect, Dahla and Loban cultivars showed the lowest peroxidise activity compared with all other cultivars. These results coulde be attributed to genetical/and environmental factors. However, since all of the studied cultivars were growing in the same district (Al-madinah Al-munawwarah) and also stored at similar conditions, thus, the observed variation was mainly due to genetical factor. Similarly, peroxidase activity was screened in peels of citrus cultivars (Clemente, 1998; Mohamed et al., 2008) and apple(Valderrama and Clemente, 2004). Such localization indicated that citrus peroxidase may participate in suberization byfacilitating the formation of polyphenolic matrix (Bernards*et al*., 1999). The accumulation of the polyphenolic compounds caused the browning of the fruits. Therefore, the date cultivars with highest peroxidise activity accompanied with the enhancement of the intensity of the browning (Data not shown).

The purification of peroxidase from Barni is summarized in Table (1). The purification step of ammonium sulphate precipitation increased the fold of purification 3.75 times. The elution profile of the chromatography on DEAE-cellulose (Fig. 2) showed that peroxidase activity was detected in two peaks, one cationic peroxidase I and one anionic peroxidase II. The cationic peroxidase I with the highest peroxidase activity was applied on a Sephacryl S-200 (Fig. 3). The specific activity of Sephacryl S-200 peroxidase I was 912 units/mg protein which represented 22.51 purification fold over the crude extract. The peroxidase I was proved to be pure after Sephacryl S-200 column as judged by SDS-PAGE (Fig. 4). Several studies reported a number of cationic and anionic isoperoxidases from fruits (Clemente, 1998; Valderrama and Clemente, 2004; Mohamed et al., 2008). The differences of the number of peroxidases from the same of fruit may be attributed to ecological, environmental and stage of maturity (Suzuki et al., 2006).

The molecular weight of peroxidase I by Sephacryl S-200 and SDS-PAGE procedures yielded a value of 64 kDa as monomer subunit. Different monomer subunit molecular weights had been reported for peroxidases from Raphanussativus L (wild radish) (56 kDa) (Valetti andPicó 2013), horseradish (56 kDa) (Mohamed*et al.*, 2011a), Ficussycomoruslatex (43 kDa) (Mohamedet al., 2011b), chewing stick miswak (70 kDa) (Mohamedet al., 2012) and date palm leaves (55 KDa) (Al-Senaidy and Ismael 2011). However, tetramer subunit molecular weights were reported for peroxidases from oil palm leaf (48 kDa) (Deepa andArumughan 2002) and vanilla bean (46.5 kDa) (Marquezaet al., 2008).

The substrate specificity of peroxidase I had been examined using a number of potential natural electron donor substrates (Table 2). The activity with the guaiacol was regarded as 100% activity. o-Phenylenediamine had a highest peroxidase activity (157%), whileo-dianisidine had moderate peroxidase activity. Pyrogallol, p-aminoantipyrine and catechol had low affinity toward enzyme. Similarly, o-phenylenediamine was found to be the best substrate for peroxidases from chewing stick miswak (Mohamed et al., 2012) and horseradish (Mohamed et al., 2011a). The physiological role of peroxidase I was the oxidation of some phenolic substrates. Therefore, peroxidase I may change the color of dates cv. Barni because several reports have shown that peroxidase plays an important role in enzymaticbrowning together with phenolic compounds (Francisco and Juan, 2001).

The kinetic parameters ofperoxidase I for H_2O_2, o-phenylenediamine, guaiacol and o-dianisidine were obtained by a typical double reciprocal Lineweaver-Burk plot (Figs.5,6,7 and 8). The apparent Km value ofperoxidase I for H_2O_2 was 2.17 mM/ml. Different Km values of H_2O_2 had been reported for peroxidases from *C. jambhiri* (0.5 mM) (Mohamed *et al*., 2008), *Ficussycomoruslatex* (1.2 mM) (Mohamed *et al*., 2011b) and black gram husk (43.5 mM) (Ajila andRao 2009).

Table 1: Purification scheme of peroxidase from Barni.

Step	Total protein (mg)	Total activity (units*)	S.A. (units/ mg protein)	Fold purification	Recovery (%)
Crude extract	42	1703	40.5	1	100
Amm. Sulphate precipitation	6.44	980	152.17	3.75	57.54
Chromatography on DEAE- Sepharose					
0.0mM NaCl (peroxidase I)	0.48	275	536.5	13.91	16.14
0.05 mMNaCl (peroxidase II)	0.15	10	66	1.60	0.58
Gel filtration on Sephacryl S-200 Peroxidase I	0.25	228.60	912	22.51	13.42

*One unit of peroxidase activity is defined as the amount of enzyme that increases the O.D. 1.0 per min under standard assay conditions.

Table 2.Relative activities of peroxidase I from Barni toward different substrates.

Substrate	Relative activity (%)
Guiacol	100
o-Phenylenediamine	157
o-Dianisidine	58
Pyrogallol	18.4
Catechol	11

Table 3: Effect of metal cations on peroxidase I from Barni.

Metal ions	Concentration (mM)	Relative activity (%)
Fe^{3+}	0.5	115
	1	334
Co^{2+}	2	101
	5	66
Ca^{2+}	2	90
	5	75
Cu^{2+}	2	113
	5	61
Ni^{2+}	2	115
	5	95
Fe^{2+}	2	110
	5	115
Zn^{2+}	2	95
	5	89
Hg^{2}	2	98
	5	63

The enzyme activity without metal ions was taken as 100%.

Table 4: Effect of metal chelating compounds on peroxidase I from Barni.

Chelating compound	Relative activity (%)	
	2 mM	5 mM
EDTA	79	78
Sodium oxalate	88	80
Sodium citarte	75	72
1,10-Phenanthroline	72	00

The enzyme activity without metal chelators was taken as 100%

The apparent Km values ofperoxidase I for o-phenylenediamine, guaiacol and o-dianisidine were 5, 5.55 and 20 mM/ml, respectively. Various Km values, using different electron donor substrates, were reported for peroxidases from *Brassica napus*(3.7 mMguaiacol; Duarte-Vazquez*et al*., 2001), black gram husk (4.7 mMo-dianisidine; Ajila andRao 2009), chewing stickmiswak(17.33 mMguaiacol; Mohamed *et al*., 2012) and C. jambhiri (5 mMguaiacol; Mohamed *et al.*, 2008).

Peroxidase Iwas found to have temperature optimum at 40℃, where enzyme retained 91, 76 and 49% of its activity at 25, 50 and 60℃, respectively (Fig. 9). The enzyme was completely lost of its activity at 70℃. The same temperature optimum was detected for peroxidases from horseradish (Mohamed *et al*., 2011a), chewing stick miswak (Mohamed *et al*., 2012) and Citrus jambhiri (Mohamed *et al*., 2008). The low temperature optima were detected for peroxidases from buckwheat seed (10-30℃) (Suzuki et al. 2006) and Vanilla bean (16℃) (Marqueza*et al*., 2008). The effect of temperature on stability of peroxidase I appeared to be non-linear in relation to temperature (Fig. 10). The enzyme activity was remained stable up to 40℃. The enzyme retained 89 and 60% of its activity at 50 and 60℃, respectively. The non- linearity of thermal stability was detected for peroxidases from horseradish (Mohamed *et al.*, 2011a), Citrus jambhiri (Mohamed *et al*., 2008), apple (Valderrama and Clemente, 2004), orange (Clemente, 1998) and marula fruit (Mdluli, 2005).

Generally, pH affected the enzyme activity through alteration of the ionization state of amino acids of enzymes. The effect of pH on the activity of Peroxidase I was shown in Fig. 11. Peroxidase I exhibited broad pH optimum from pH 5.5 to 6.0. More than 50% of its activity was retained at pHs from 4.5 to 6.5, where the enzyme kept a good activation about 43 and 49 % of its activity at pHs 7 and 7.5, respectively. A broad pH optimum ranged from pH 5.5 to 7.0 was also detected for peroxidase from *Ficussycomoruslatex* (Mohamed *et al*., 2011b).The highest acidic and alkaline pH optima were detected for peroxidases from marula (pH 4.0) (Mdluli, 2005) and buck wheat (pH 9.0) (Suzuki et al., 2006). However, the release of heme group from the active site of peroxidase was occurred at lower and higher pH and lead to the loss in activity (Lopez and Burgos, 1995).

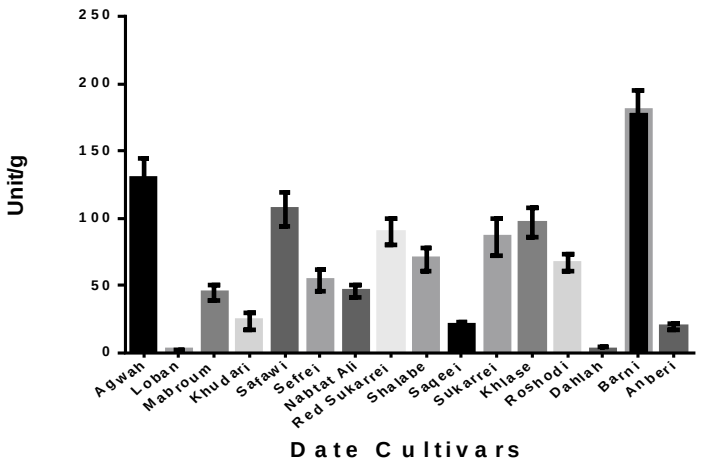

Fig.1: Screening of peroxidase activity in different cultivars of Saudi dates palm.

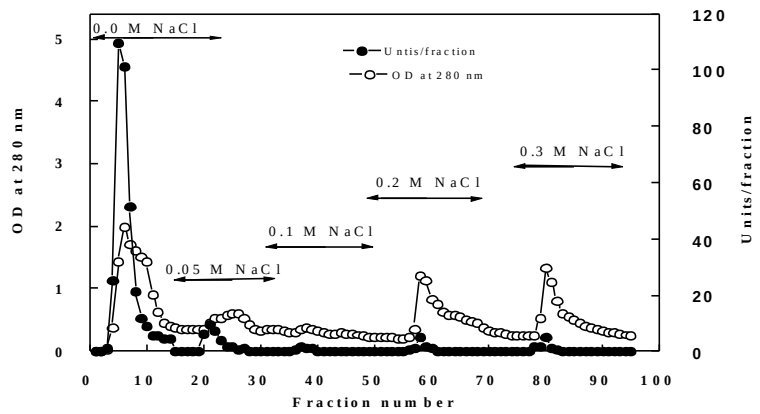

Fig. 2: A typical elution profile for the chromatography ofperoxidase from dates palm cv. Barni on DEAE-Sepharose column (10 x 1.6 cm i.d.) previously equilibrated with 20 mMTris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions.

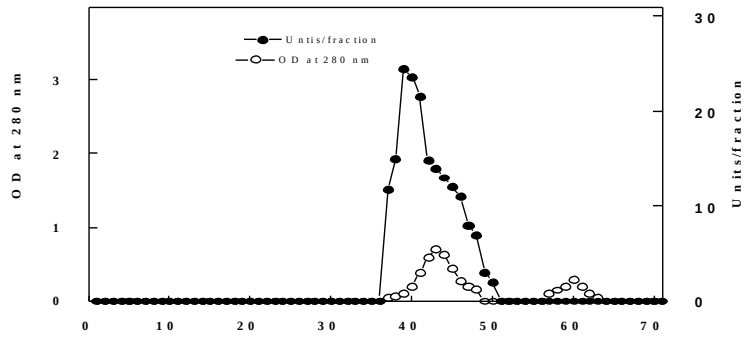

Fig.3: Gel filtration ofperoxidase I from dates palm cv. Barni from DEAE-Sepharose fraction on Sephacryl S-200 column (90 x 1.6 cm i.d.). The column was equilibrated with 20 mMTris-HCl buffer, pH 7.2 at a flow rate of 30 ml/h and 3 ml fractions.

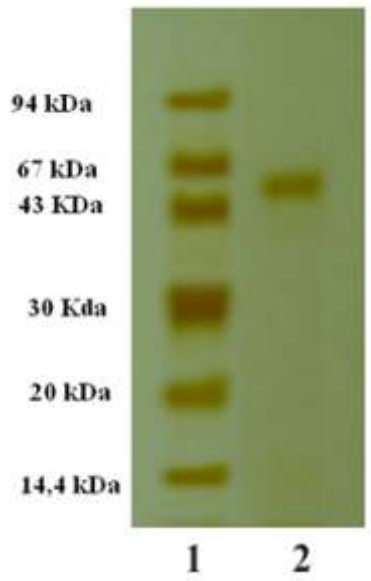

Fig. 4: SDS-PAGE for homogeneity and molecular weight determination of peroxidase I from cv. Barni. 1- Protein markers; 2- Sephacryl S-200 peroxidase I.

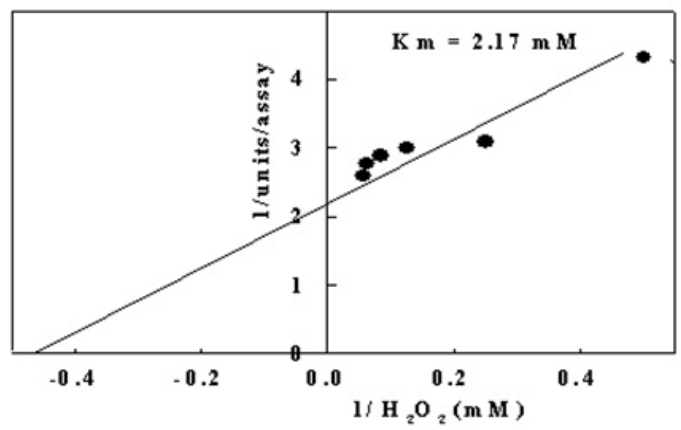

Fig.5:Lineweaver-Burk plot relating ofperoxidase I from dates palm cv. Barni reaction velocities to H2O2 as substrate concentration.

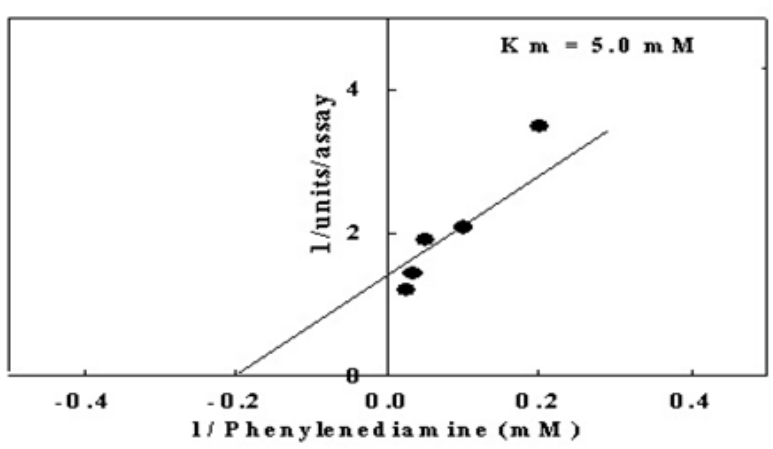

Fig. 6:Lineweaver-Burk plot relating ofperoxidase I from dates palm cv. Barni reaction velocities to o-phenylenediamine as substrate concentration.

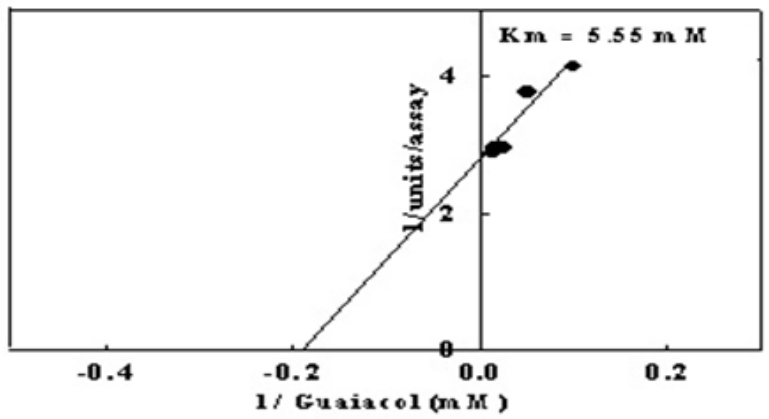

Fig.7:Lineweaver-Burk plot relating ofperoxidase I from dates palm cv. Barni reaction velocities to guaiacol as substrate concentration.

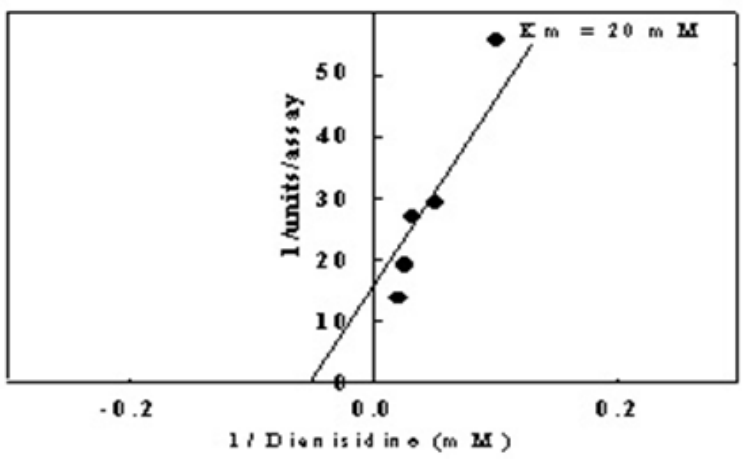

Fig.8:Lineweaver-Burk plot relating ofperoxidase I from dates palm cv. Barni reaction velocities to o-dianisidine as substrate concentration.

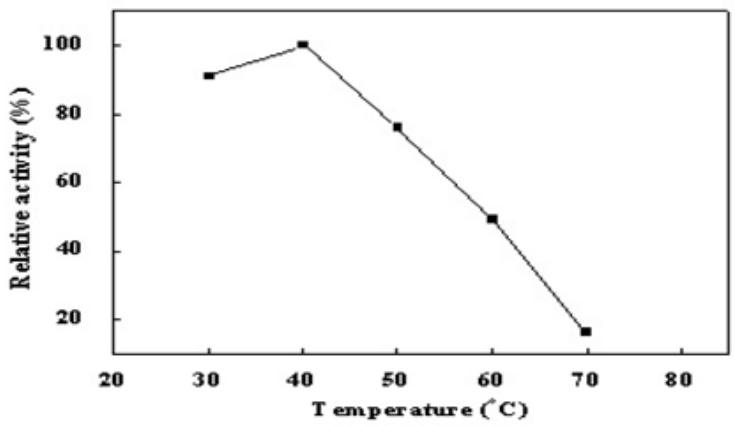

Fig. 9: Temperature optimum ofperoxidase I from dates palm cv. Barni. The enzyme activity was measured at various temperatures using the standard assay method as previously described.

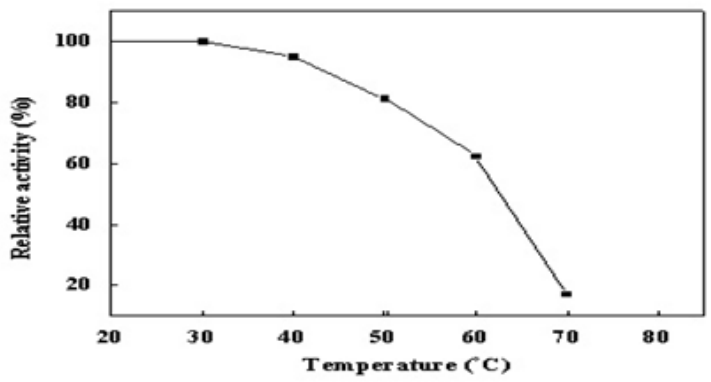

Fig.10: Thermal stability ofperoxidase I from dates palm cv. Barni. The assay reaction mixture was pre-incubated at various temperatures for 15 min prior to substrate addition, followed by cooling in an ice bath. Activity at zero time was taken as 100% activity.

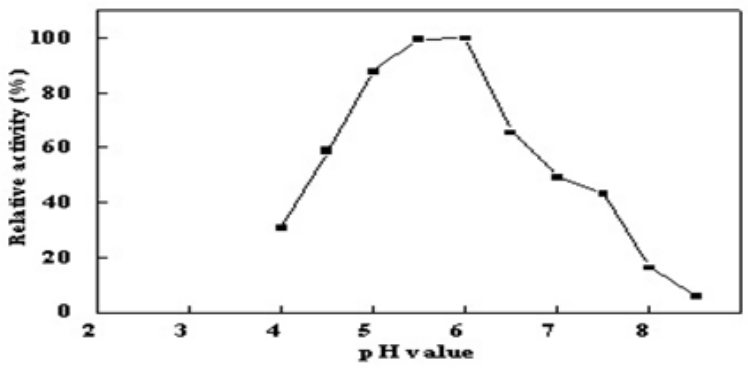

Fig.11: pH optimum ofperoxidase I from dates palm cv. Barni.

The effect of different metal ions on peroxidase I was examined (Table 3). The metal ions were used at the concentrations of 2 or 5 mM expect of Fe^{3+} used 0.5 or 1 mM. At 1 mM concentration, Fe^{3+} was able to greatly enhance the activity of peroxidase I by 334%, while Fe^{2+} at 2 and 5 mM slightly enhanced the activity. Cu^{2+} and Ni^{2+} at 2 mM also slightly enhanced activity of peroxidase I. The other metals tested at 2 mM had no effect on enzyme activity, while at 5 mM moderate inhibitory effect on enzyme activity was detected. Similarly, Fe^{3+} at concentration of 1 mM enhanced the activity of both m-POD-I (339%) and m-PODII (328%) from Metroxylonsagu (Onsa*et al*.,2004). At 5 mM concentration, Fe^{3+} was able to enhance the activity of peroxidase from black gram husk to 145% (Ajila andRao, 2009). Fe^{3+} is considered essential for the activity of most plant peroxidase enzymes as it is involved in binding of H2O2 and formation of compound I (Whitaker, 1994).

The effect of different concentration of EDTA, sodium citrate, sodium oxalate and 1,10-phenanthroline on

peroxidase I wasstudied (Table 4). All the metal chelators examined caused partially inhibitory effects on activity of peroxidase I, except of 5 mM 1,10-phenanthroline caused completely loss of its activity. EDTA, sodium oxalate and sodium citarte were unable to fully combine with Fe^{3+} atom found in the peroxidase active center. EDTA had moderate inhibitory effect on peroxidase from vanilla bean (Marqueza*et al.*, 2008), while Fujita*et al.*(1995) reported unsuccessful inhibition of peroxidase by EDTA. EDTA, sodium oxalate and sodium citarte caused moderate inhibitory effects on peroxidase from chewing stick miswak (Mohamed et al., 2012).

CONCLUSION

From the obtained results in this study, it could be concluded that the highly fluctuations in the levels of peroxidase activity in different cultivars of Saudi dates palm in tamer stage. The date cultivars with highest peroxidise activity accompanied with the enhancement of the intensity of the browning. Barni peroxidase I was characterized by (i) a low Km value suggesting high enzyme–substrate affinity, (ii) an acidic broad pH optimum 5.5-6.0, (iii) temperature optimum at 40°C (iv) a broad substrate specificity on a large number of potential natural electron donor substrates, and (v) a high tolerance towards a large number of metal ions. From these findings, Barni peroxidase I could be considered a beneficial enzyme for several biotechnological applications. Also, the characterization of Barni peroxidase I could be useful in dates palm processing, where peroxidae caused changes in texture, flavor, and color for fruits and vegetables.

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