

Thermostable Characteristics of Peroxidase from Leaves of Arabian Palm Date (*Phoenix dactylifera* L.)

Majed S. Alokail and Mohammad A. Ismael
 Dept. of Biochemistry, College of Science,
 King Saud University,
 P.O. Box - 2455, Riyadh 11451,
 Saudi Arabia
 E-mail: msalokail@yahoo.com

Abstract. Peroxidases are enzyme that are widely distributed in the living world including plants and that are involved in many physiological processes, including the conversion of H_2O_2 to H_2O . We investigated here the enzyme assay, optimum pH, and temperature, heat inactivation, and determination of V_{max} and K_m of peroxidase enzyme from leaves of the Arabian palm (*Phoenix dactylifera* L.) tree. Our results show that the maximum enzyme activity was observed between pH 5 and 8, and the optimum catalytic pH value was between 30 and 50 °C. Moreover, the apparent V_{max} and K_m was 20.43 $\mu\text{mol}/\text{min}/\text{ml}$, and 1.018 mM, H_2O_2 respectively. Heat inactivation studies, showed that the enzyme was stable at 60 °C during one hour incubation. These results suggest that the Arabian date palm peroxidase enzyme appears to be a highly stable enzyme, and might have biotechnological applications since the Arabian date palm tree grow during summer months in high temperature in Arabian Peninsula.

Key word: Peroxidase; Arabian date palm, *Phoenix dactylifera* L. thermal stability.

Introduction

Peroxidase (EC 1.11.1.7; donor:hydrogen-peroxide oxidoreductase) is an oxidoreductase that is known to catalyse the oxidation of numerous substances through the associated reduction of hydrogen peroxide. $[H_2O_2 + \text{Colorless Dye}_{(\text{reduced})} \xrightarrow{\text{peroxidase}} H_2O + \text{Colored Dye}_{(\text{oxidized})}]$ (Dawson, 1988; Wallace and Fry, 1999). Given the ubiquitous presence of peroxidase throughout nature (Vámos-Vigyázó, 1981) and the number of substrates that may potentially be oxidized in the presence of peroxidase, it seems unlikely that a single peroxidase will catalyse a single specific reaction *in vivo*. In plants, the main roles of peroxidase are attributed to lignification and suberization (Müsel *et al.*, 1997; Quiroga *et al.*, 2000), cutin deposition in outer aerial epidermal

layers (Ferrer, *et al.*, 1991), defence against pathogen attack (Lagrimini, *et al.*, 1993) and the possible cross-linking of cell wall components (Brownleader, *et al.*, 1999). Insolubilization of structural proteins has been believed to strengthen the cell wall barrier against pathogen attack., and peroxidase catalysed cross-linking in cell walls is thought to result from the formation of diferuloyl bridges pectin residues, and isodityrosine bridges between hydroxyproline-rich extension molecules (Hatfield, *et al.*, 1999).

Several peroxidases have been isolated, sequenced and characterized. They has essentially been classified in three classes, supported in the first instance by comparison of amino acid sequence data and confirmed by more recent crystal structure data (class I, intracellular prokaryotic peroxidases; class II, extracellular fungal peroxidases, and class III,

secretory plant peroxidases) (Dunford 1991; Welinder 1992). This suggests that different peroxidase molecules may exhibit different interactions with the various constituents of the cellular matrix. In addition, the plant peroxidase is an oxidoreductase catalyze oxidation of abundant electron donors with hydrogen peroxide where E, EI, and EII are native enzyme and its compounds I and II, respectively; S and P are substance and product of its one-electron oxidation (Dunford 1991; Welinder 1992). all oxidizing a broad range of organic and inorganic substrates at the expense of H₂O₂.

- (i) (Resting state) E + H₂O₂ ----->EI+ H₂O
 (ii) EI + S -----> EII + P
 (iii) EII+ S+H⁺-----> (Resting state) E+ H₂O+P

The most plant peroxidase is the one obtained from horseradish roots (HRP), which is also the most commercially available one. However, other plant species may provide peroxidases with similar or even improved properties. Therefore, the availability of highly stable and active peroxidases from sources other than horseradish roots would go a long way toward the development of a catalytic enzyme with broad commercial and environmental possibilities (Krell 1991). Several publications have addressed the study of the conformational stability of peroxidases, but to date our understanding of their folding mechanism remains contradictory and unclear (Chattopadhyay and Mazumdar 2000; Pina *et al.*, 2001). Factors affecting conformational stability have been studied most intensively in proteins under reversible conditions (Freire 1995).

Date palm (*Phoenix dactylifera* L.) a long-living monocotyledon plant and this species classification includes oil palms, Coconut palms, and Washingtonia. Traces of cultivation of the date palm tree are found four thousand years ago, from its emergence in the Tigris and

Euphrates valleys (the Fertile Crescent) area near the Arabian Gulf. Date palm is the most economic importance crop in Saudi Arabia. Since previous studies highlighted the environmental importance of plant peroxidase, there is no information published about Arabian palm date peroxidase. We describe here the optimum pH, temperature, heat inactivation, and determination of V_{max} and K_m of Arabian palm date peroxidase.

Materials and Methods

Materials

Leaves of Arabian date palm (Arabian species name is Sikkary) were collected and prepared as described previously (Rodriguez, *et al.*, 2002). Only fresh green leaves without any yellow spots were used in this work. Tris, Guaiacol, (NH₄)₂ S₂O₈ and H₂O₂, were from Merck (Darmstadt, Germany).

Preparation of palm leaf extract

The leaves of Arabian date palm trees approximately 15 cm in length were cut into pieces and homogenized in cold 10 mM sodium phosphate buffer (pH 7.0) (Rodriguez, *et al.*, 2002). The homogenate was centrifuged at 20,000 xg for 15 min at 4 °C, then the centrifugation pellet was discarded.

Enzyme assay

Peroxidase activity was measured in the supernatant from Arabian date palm leaves were measured using 10 µl of enzyme solution in 100 mM phosphate buffer (Ph 6.0), containing 8 mM guaiacol and 3 mM H₂O₂ as substrates, and the absorbance change at 470 nm was measured at 25 °C (Rodriguez, *et al.*, 2002). One unit of activity (U) is defined as the amount of peroxidase oxidizing 1 µmole of substrate per min under standard conditions.

Optimum pH

Peroxidase activity was measured at different pH and the optimum pH was determined by using 50 mM Na-phosphate and

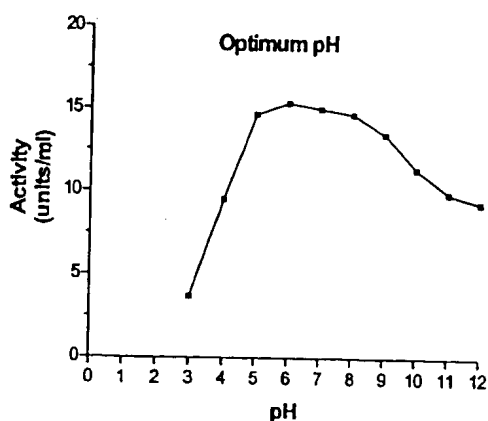


Fig 1. Optimum pH determination. Peroxidase activity in Date palm leaf has been measured in 50 mM phosphate buffer and Na acetate buffer with various pH (3-12). The maximum enzyme activity were observed pH 5 and 8.

sodium acetate buffers.

Optimum Temperature

Peroxidase assay at different temperature (20°C–80°C) was monitored by incubating the enzyme at the mentioned temperatures and H₂O₂ reduction was determined at 470 nm.

Determination of V_{max} and K_m

V_{max} and K_m was determined by using different concentrations of H₂O₂. The reaction mixture included Na-phosphate buffer pH 6.0, 8 mM guaiacol and various concentrations of H₂O₂. Increase in absorbance was measured at 470 nm.

Heat Inactivation

100 µl of enzyme extract was incubated in a water bath at 60 °C for a period of 10, 20, 30, 40 and 60, minutes. Once the required heating time was reached 10 µl aliquots were immediately transferred to ice, and the activity of each sample was then measured.

Results and Discussion

This report identified novel properties of peroxidase enzyme in Arabian date palm tree (*Phoenix dactylifera* L.). This study was able to investigate the optimum pH and temperature, the enzyme V_{max} and K_m values and the heat inactivation. The data shown interesting

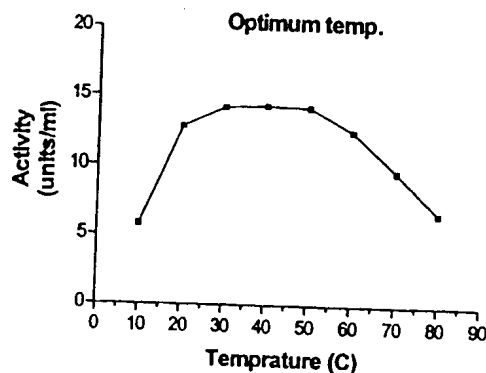


Fig 2. Peroxidase optimum temperature determination in date palm tree. The enzyme activity was measured between 20 °C to 80 °C, and the H₂O₂ reduction was measured at 470 nm. The maximum enzyme activity has been shown at 30 °C, 40 °C, and 50 °C.

enzyme condition with heat stability reached 60 °C. The activity was observed between pH 5 and 8 (Figure 1), and the maximum enzyme activity has been shown optimum temperature at 30 °C, 40 °C, and 50 °C (Figure 2). Moreover, the V_{max} and K_m values were determined at 20.43 µmol/min/ml, and 1.018 mM, respectively (Figure 3). The peroxidase inactivation experiments have shown that the enzyme is stable at 60 °C in various incubation periods (Figure 4). Although the tree peroxidases in general and in date palm in specific almost nothing is known about their physiological importance, their structural characteristics, or the mechanism of covalent bond formation.

Most higher plants can quickly activate various defense systems to protect themselves from pathogen attack, injury, or other forms of stress. Among them, insolubilization of structural proteins has been believed to strengthen the cell wall barrier against pathogen attack. Peroxidases are ubiquitous enzymes that catalyze oxidation of cellular components in the presence of H₂O₂. Most higher plants contain a number of peroxidase isozyme, which can be classified into two (anionic and cationic) or three (anionic, neutral,

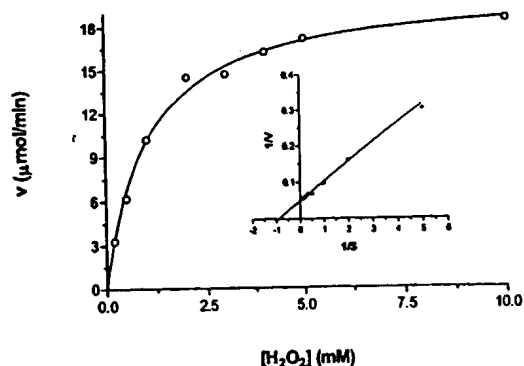


Fig 3. The determination of Arabian date palm peroxidase V_{max} and K_m by Michaelis-Menten equation. Values were determined using different concentrations of H_2O_2 . The reaction mixture included Na-phosphate buffer pH 6.0 and 8 mM guaiacol with various concentrations of H_2O_2 . The absorption was measured at 470 nm. The V_{max} and K_m values were 20.43 $\mu\text{mol}/\text{min}/\text{ml}$ and 1.018 mM, respectively. The inset curve is Lineweaver-Burk plot.

and cationic) subgroups according to their isoelectrophoretic mobilities, and these isozymes exist in cytosol, chloroplast, vacuole, and cell wall (Asada 1992). Their physiological roles have been extensively investigated, and it has been demonstrated that they catalyze a variety of important reactions, such as indole-3-acetic acid catabolism, lignin biosynthesis, suberization of cell wall and detoxification of H_2O_2 (Grisebach 1981; Christensen *et al.*, 1998).

In recent study, peroxidase has been isolated from the leaves of the African oil palm tree *Elaeis guineensis* (Sakharov *et al.*, 2000). This peroxidase shows a characteristic spectrum for haem-containing proteins, with a Soret maximum at 403 nm. Its molecular mass as estimated by SDS/PAGE is 57,000 daltons which is higher than the values published for other plant peroxidases (Dunford, 1991), probably because of the higher degree of glycosylation. It has also been found that African oil palm tree *Elaeis guineensis* peroxidase, similar to peroxidases that have been detected in the sweet potato, royal palm tree, tobacco, and tomato (Andrews *et al.*, 2002). The data have shown that the palm date

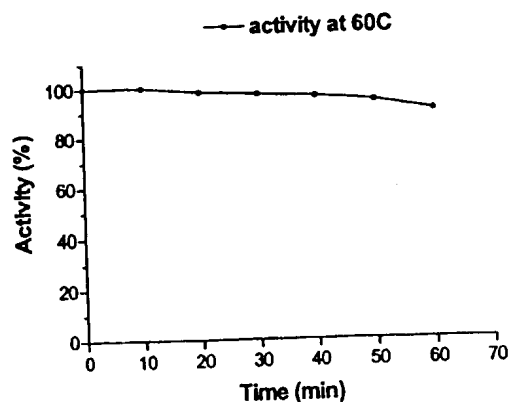


Fig 4. Heat inactivation of Arabian palm date peroxidase. The enzyme extract was incubated in a water bath at 60 °C for 10,20,30,40 and 60 minutes. The peroxidase activity of each time was determined at 470 nm.

peroxidase activity was observed between pH 5 and 8, and this range of pH has been reported previously by several studies in different kinds of plant. In cases of horseradish peroxidase and beans cell wall, the pH optimum was 8.5 and 7.2, respectively (Bolwell *et al.*, 2002). Similar to our data in maximum stability of Arabian palm date peroxidase, Sakkarov (2001) have reported that the heme-peroxidase from palm tree leaves *Elaeis guineensis* is stable over a broad pH-range, maximum stability being found at pH 7.0. Compared with our data, African oil palm tree *Elaeis guineensis* peroxidase acidic (pH 2.0) and alkaline (pH 12.0) conditions, showed a lower stability but remained highly stable enzyme, losing not more than 20% of its initial activity for 30 min at 25°C (Sakharov, 2001). The observed inactivation temperature of peroxidase has been reported to be 90.5°C in Soybean seed coat peroxidase, 81.5°C in horseradish peroxidase C, and that of *Coprinus cinereus* peroxidase (a class II peroxidase from the fungus *C. cinereus* with a similar activity) is 65°C (McEldoon and Dordick 1996). The present work on peroxidase activity of Arabian palm date leaves confirms the above findings and supports the notion that peroxidase has thermostability (Figure 4).

In addition, peroxidase can alter the cell

wall properties by promoting the cross-linking between molecules like lignin, suberin, proteins, hemicelluloses, and ferulic acid (Espelie *et al.*, 1986). The peroxidase activity could suggest that insolubilization of cell wall proteins had already been observed as a result of osmotic stress and was considered to contribute to the adjustment of cell wall elasticity despite the water loss (Marshall *et al.*, 1999). In fact, the physiological significance of peroxidase activity under drought could be related to changes in the cell wall properties potentially important for the stem in order to cope with the stress (Trudel *et al.*, 1998) and to hydrolyze water-soluble and -insoluble complex β -1,3-glucans (Grenier *et al.*, 1999). Since water drought causes the formation of active oxygen species, an additional function of the increased peroxidase activity could be the protection against oxidative damage (Tabaeizadeh, 1998).

Furthermore, it was apparent that peroxidase activity was mainly located in the inner regions of the cell wall with occasional activity observed within the middle lamella (Thompson *et al.*, 1998). The presence of 'wall-bound peroxidase activity in mature fruit confirms earlier findings and supports the notion that peroxidase mediated 'stiffening' of the exocarp cell walls leads to the cessation of fruit growth (Thompson *et al.*, 1998; Andrews *et al.*, 2000). The role of a highly anionic peroxidase in wounded potato tubers and tomato fruits has been previously reported and appears to mediate deposition of aromatics in the cell wall (Espelie *et al.*, 1986; Andrews *et al.*, 2002). The presence of such an aromatics normally associated with lignin and suberin deposition may also provide resistance to pathogen infection (Lulaiand and Corsini, 1998). Relatively few pathogens affecting the fruit of tomato appear to gain entry into the fruit through the fruit skin. Those that do, appear to enter when the fruit are immature and the skin is thin and poorly developed or

through wound or growth cracks in older fruit. Therefore, the protective barrier presented by the fruit skin may be fundamental to resistance against localized pathogen attack (Watterson, 1986).

In conclusion, the thermal stability characteristics of Arabian palm date (*Phoenix dactylifera* L.) peroxidase has been investigated in this work. The results suggested that it is likely that the enzyme has potential roles in Arabian date palm since these trees grow in severe high temperature during summer period (between 35 to 50°C). It is clear that Arabian palm date peroxidase is substantially more thermostable than we expected. Since the plant peroxidases is little is known about their physiological importance, our finding of unique high thermostability of Arabian palm date peroxidase promises good perspectives for this peroxidase in Arabian palm date peroxidase biotechnological applications.

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خواص المقاومة الحرارية لإنزيم البيروكسيديز من أوراق النخيل العربي (*Phoenix dactylifera L.*)

ماجد صالح العقيل و محمد الحسن إسماعيل
قسم الكيمياء الحيوية، كلية العلوم، جامعة الملك سعود
ص.ب. ٢٤٥٥، الرياض ١١٤٥١
المملكة العربية السعودية.

المخلص : يوجد إنزيم البيروكسيديز بشكل واسع في معظم الكائنات الحية ومن بينها النباتات حيث إنه يدخل في العديد من المسارات الفسيولوجية، مثل تحويل الـ H_2O_2 إلى H_2O . فلقد تم في هذا البحث دراسة الطرق الإنزيمية، والأس الهيدروجيني الأمثل، ودرجة الحرارة المثلى، وقياس التنشيط الحراري، والمعايير الحركية لإنزيم البيروكسيديز في أوراق شجر النخيل العربي . فقد أظهرت النتائج بأن أقصى فاعلية لهذا الإنزيم قد شوهدت بين الأس الهيدروجيني ٥ و ٨ وعند درجة حرارة ٣٠ - ٥٠ درجة مئوية. بالإضافة لذلك فقد حدد ثابت ميكليس والمعروف بالـ K_m وثابت السرعة القصوى والمعروف بالـ V_{max} عند ١,٠١٨ مل مولار و ٢٠,٤٣ ميكرومولار/دقيقة/مل على التوالي. وأظهرت النتائج الخاصة بالتنشيط الحراري على ان نشاط الإنزيم ظل مستقرا عند ٦٠ درجة مئوية وعلى مدى ساعة كاملة. وقد تبين من هذه الدراسة بأن إنزيم البيروكسيديز في أوراق شجر النخيل العربي ذو ثباتية حرارية عالية، وإن هذا الإنزيم ربما يكون له أهمية في الإستعمالات التقنية الحيوية لكون شجر النخيل تتحمل درجات حرارة عالية خلال أشهر الصيف في شبه الجزيرة العربية.