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PURIFICATION AND CHARACTERIZATION OF PEROXIDASE FROM DATE PALM (*PHOENIX DACTYLIFERA* L.) CALLUS CV. BARHEE

Mostafa, M. A[1]; Abd Elbaky, M. A[2]; Abbas, M. S[2] and Soliman, A. S[2]

[1] Cenrtal Laboratory for Date Palm Research and Development, Agriculture Research Center, Giza, Egypt
[2] Natural Resources Department, Institute of African Research and Studies, Cairo University, Giza, Egypt

ABSTRACT

Peroxidase (POD, E.C 1.11.1.7) is one of an enzymatic defense system against active oxygen species (AOS) in date palm (*Phoenix dactylifera* L.), especially during callus stage. This study aimed to extract peroxidase (POD) from date palm (cv. Barhee) callus tissue for biochemical characterization. In the crude extract of callus peroxidase activity reached (161.14 U). Partially purified POD with 30% ethanol reached 7.14 purification fold, whereas it increases with Sephadex G-100 to reach 61.77. The molecular weight of purified POD was 43 kDa while the optimum pH and temperature were 6.5 and 40°C, respectively. Kinetic constants Km and Vmax for guaiacol was 0.4 mg/reaction and 0.87U.min^{-1}, respectively while for H_2O_2 was 0.24 mM/reaction and 0.44 U.min^{-1}. Browning phenomenon in Barhee callus could be referred to the increase of POD activity always accompanied with phenolic compounds oxidation. Determination of optimum conditions for POD from Barhee date palm callus could control the browning phenomenon in additions could be a source as a natural product from Barhee date palm callus for another application.

Keywords: Callus; Peroxidase; Barhee; *Phoenix dactylifera* L.; purification.

INTRODUCTION

Active oxygen species (AOS) such as hydrogen peroxide, superoxide radical, and hydroxyl radical capable of causing oxidative damage to the cell. In higher plants, there are two main defense system against active oxygen species, an enzymatic and non-enzymatic type. The enzymatic system is carried out by a series of redox enzymes generally catalyzing electron transfer to the AOS using low molecular weight antioxidant compounds (e.g. ascorbate, tocopherol, glutathione, phenols and flavonoids) as electron and proton donors **(Adam *et al*., 1995, Bestwick *et al*., 2001, Gholizadeh and Kohnehrouz, 2010).** Peroxidase (POD) (E.C 1.11.1.7) working as anti-oxidant in date palm defense system **Zein Eldin and Ibrahim (2015a)**It is also involved in many physiological processes such as biotic and abiotic stresses, biosynthesis of lignin and preventing from the harmful effect of AOS. As an anti-oxidant; POD acts on the oxidation of phenolic substances in the presence of H_2O_2, where hydrogen peroxide acts as a receptor for the dissolved hydrogen (phenols), amino acids or alcohols **(Vicuna, 2005, Singh *et al.*, 2016)**.

Date palm (*Phoenix dactylifera* L.) callus initiation and formation is very slow process and takes along time , that made callus facing many problems browning, contamination, vitrification and somaclonal variation **(Saker *et al*., 2000, Alkhateeb, 2008, Al-Khayri and Naik, 2017).** Gradually, the callus loses its vitality and (a lot of plant source) was lost in this stage.

This study aimed to detrmine and characterize biochemically peroxidase during callus stage, espicially the optimum conditions for POD to control browning during callus formation and suitability for further development.

MATERIALS AND METHODS

1. Plant material:

Explants were isolated according to **Zein Eldin and Ibrahim (2015b)** and transferred to callus induction media which consisted of MS **Murashige and Skoog (1962)** powder plus vitamins (Nicotinic acid, Pyridoxine-HCl, Thiamine-HCl and Myo-inositol) 4.4 g/L, 50 g/l sucrose + 200 mg/l L-glutamine + 200 mg/l KH_2PO_4-$2H_2O$ + 10 mg/l

2,4-D (2,4-Dichlorophenoxyacetic acid) + 3 mg/l 2iP (isopentenyl adenine) + 1g/l activated charcoal + 50 mg/l adenine sulphate.

2. Enzyme extraction:

Fresh callus (1g) was ground within liquid nitrogen using mortar and pestle and homogenized with 4ml of cold sodium phosphate buffer (100mM, pH=7) containing 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1 mM EDTA. The homogenate was centrifuged at 2268g at 4ºC for 15min.The supernatant was used for measurement of crude peroxidase activity.

3. Purification of POD:

The purification protocol consisted of (Ethanol, Gel filtration chromatography).

a- Ethanol treatment:

Crude callus extract was treated with varied concentrations of ethyl alcohol (cooled at 5°C) according to **Munir *et al*. (2015)** . Certain volume of cold ethanol was slowly added while stirring to cold enzyme solution (kept in an ice-salt bath) until the required concentration of ethanol was reached. After removing the precipitated fraction by centrifugation at 2012 g for 10 minutes in a cooling centrifuge. Further ethanol was added to the supernatant fluid and the process was repeated to obtain several enzyme fractions, i.e. 20, 30, 40, 50, 60, 70, 80, and 90% of ethanol. Protein content and enzyme activity of each fraction were determined.

b- Gel filtration chromatography:

Highest purified ethanol fraction was obtained and applied on a gel filtration chromatography Sephadex G-100 (fractionation range 4–150kDa) equilibrated with 150 mM phosphate buffer (pH 7.0) at 4°C for 24 h and eluted with 0 to 0.5 M linear gradient of NaCl at a flow rate of 0.25 mL/min. The obtained fraction every 3 ml was used to determine POD activity and soluble proteins concentration.

4.**Soluble proteins determination**

The soluble protein in enzyme's crude extract was quantified by Bradford method according to **Bradford (1976)** using bovine serum albumin as a standard.

Peroxidase activity assay

Peroxidase activity was quantified by the method of **Hemeda and Klein (1990)**. The assay mixture (100 ml) contained 10 ml of 1% (v/v) guaiacol, 10 ml of 0.3 % H_2O_2 and 80 ml of 50mM phosphate buffer (pH=6.6). Volume of 100 µl of crude enzyme was added to 2.9 ml of the assay mixture to start the reaction. The absorbance was recorded every 30s for 3 min at 470 nm using spectrophotometer. The rate of change in absorbance per minute was calculated and one unit of enzyme was expressed as $\Delta OD = 0.01$. POD activity was expressed as unit.mg^{-1} protein.

Molecular weight determination

Sodium dodecyl sulphate (SDS) -PAGE was used for determination of molecular weight (MW) of POD from date palm (cv. Barhee) formatted callus according to the method of **Al-Senaidy and Ismael (2011)**. Discontinuous electrophoresis was conducted by using 12% SDS- polyacrylamide gel and protein molecular weight marker consisted of purified protein (116.0 kDa galacosidase, 97.4 kDa phsophorylase b, 66.2 kDa bovine serum albumin, 37.6 kDa alcohol dehydrogenase and 28.5 kDa carbonic anhydrase). The molecular weight of POD was determined by a calibration curve (log molecular weight of the standards vs. retention factor value).

Optimum pH

The purified POD was tested in pH range (5.0 - 8.0) to determine the optimum pH for POD activity. Phosphate buffer was used to adjust pH from 5-7, whereas basic Tris-buffer was used to adjust pH from 7 to 8.

Optimum temperature

Enzyme activity was determined for purified POD at temperature range from 10 to 70^{o}C) to determine the optimum temperature for POD enzyme.

POD substrate concentration and kinetic constants (K_m and V_{max})

POD substrate concentrations were determined for both guaiacol concentrations, i.e. 0.1, 1, 2, 3, 4 and 5 mM and for H_2O_2 concentrations, i.e. 0.1, 0.5, 1, 1.5, 2 and 2.5 mM. Data from substrate concentration effect on enzyme activity was applied to determine POD enzyme kinetic constants, i.e. K_m (Michaelis constant) and V_{max} (the maximum reaction velocity) as described by Woolf plot **Haldane**

and Stern (1932). This was attained by plotting the substrate concentrations, divided by velocity ([S]/v) versus the substrate concentration [S], which is known as Woolf plot. The vertical and the horizontal intercepts resemble K_m / V_{max} and K_m, in the same order were calculated.

Statistical analysis

The collected data were calculated as means from three replicates and were analyzed using SPSS statistical software (IBM SPSS Statistics, version 23). The differences between treatments were compared using one-way analysis of variance (ANOVA) according to method of **(Tamhane and Methods, 1977)**, Post hoc LSD test was also performed at $p \leq 0.05$.

RESULTS AND DISCUSSION

Callus of date palm (*Phoenix dactylifera*. L cv. Barhee) was successfully formed after culturing on modified MS medium for 35-40 weeks **(Fig.1)**

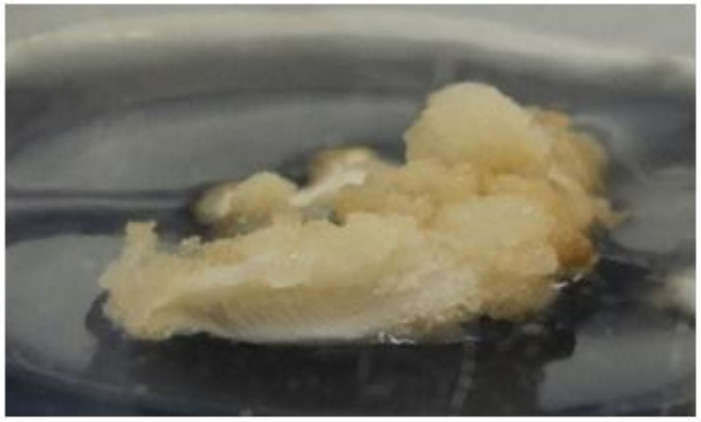

Fig.1. Date palm (*Phoenix dactylifera*. L cv. Barhee) callus formatted from explant cultured on MS basal medium.

Peroxidase extraction and purification:-

Data in **Table (1)** showed that crude extract of POD from date palm (cv. Barhee) formatted callus showed activity (161.14U), protein content (74.71mg) and specific activity (2.16 U mg^{-1}protein). Fraction precipitation method with ethanol (20-30%) showed an increase in purification with 7.14 time of purification fold, whereas its protein content decreased to 3.06 mg about (4.09%) of recovered protein in ethanol fraction compared with (74.71 mg) in crude extract, while the POD activity decreased to (47.2U) about (29.29 %) of recovered activity. Ethanol fraction was obtained for purification gel filtration chromatography. The use of Sephadex G-100 was reflected in the very low protein content (0.341mg) about (0.46 %) of recovered protein and POD activity was (45.5U) about (28.24 %) of recovered activity , that's cause a higher value of POD specific activity (133.43 U.mg^{-1}protein) and (61.77) time of purification fold. **Altın *et al.* (2017)** demonstrated that peroxidase purified from wheat (*Triticum aestivum* ssp. vulgare) with ammonium sulfate 50–60% that showed about 51 purification fold while Sephadex G-25 increase the purity to reach about 284 purification fold. **Singh *et al.* (2012)**succeeded in purifying the POD from the leaves of *Sapindus mukorossi* with acetone to four times of purification fold and increased purification with ion exchange to 14 times purification fold.

Table (1): Data of the crude peroxidase and the purified peroxidase with ethanol and Sephadex G-100

	Protein content (mg)	Recovered protein (%)	Total activity (U)	Recovered activity (%)	Specific activity (U. mg^{-1} protein)	Purification fold
Crude POD	$74.71^{a}\pm$ 1.29	$100^{a}\pm$ 0.00	$161.14^{a}\pm$ 6.96	$100^{a}\pm$ 0.00	$2.16^{c}\pm$ 0.09	$1^{c}\pm$ 00
Ethanol (20-30%)	$3.06^{b}\pm$ 0.29	$4.09^{b}\pm$ 0.43	$47.2^{b}\pm$ 1.33	$29.29^{b}\pm$ 1.21	$15.4^{b}\pm$ 0.56	$7.14^{b}\pm$ 0.2
Sephadex G-100	$0.341^{c}\pm$ 0.06	$0.46^{c}\pm$ 0.03	$45.5^{bc}\pm$ 0.4	$28.24^{bc}\pm$ 2.39	$133.4^{a}\pm$ 2.89	$61.77^{a}\pm$ 16.78
LSD at 0.05	0.68	0.99	3.27	5.53	6.41	33.56

Data represent the means ± SE calculated from three replicates. Different letters refer to significant differences at ($P \leq 0.05$).

POD molecular weight (MW) :

Purified POD after sephadex gel filtration was used for molecular weight determination with SDS –PAGE as presented in **Fig (2)** POD from date palm (cv. Barhee) callus indicates a monomer band with MW between 37.5 and 66.2 kDa. The exact MW of POD using calibration curve (log molecular weight of the standards vs. retention factor values) was 43 kDa. This result agreed with the result obtained by **Kanayama *et al.* (2002)** who found that POD from *Aspergillus terreus* had MW 43kDa. **Duarte-Vázquez *et al.* (2001)** reported that the MW for POD from Turnip (*Brassica napus* L.) was 36 kDa.

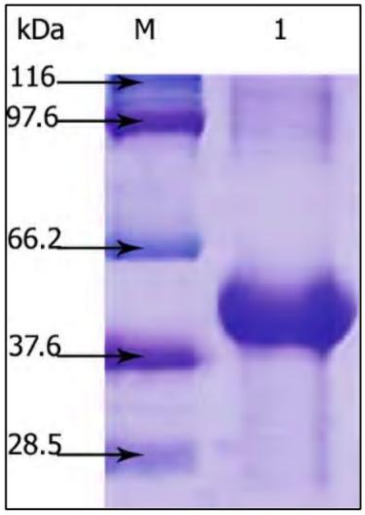

Fig (2) SDS-PAGE of peroxidase purified from date palm (cv. Barhee) callus. Molecular weight markers (Lane M), and gel filtration (Lane 1).

Optimum pH :

The purified POD with highest purification fold was tested at different pH range from pH 5 to 8 for determination of optimum pH for POD enzyme.

From the data in **Fig (2)** ; the purified POD showed activity at pH range (5.0 – 8.0). The highest POD activity was found from (6.0pH) to (7.0pH) and the optimum was at (6.5pH) with POD activity (3.03U/ml). The obtained result was in harmony with **Erdem *et al.* (2015)** who showed that the maximum activity of POD from cabbage (*Brassica oleracea* L. var. capitata) was at pH 6.5, while does not agree with **Zhang and Xingfeng (2015)** who reported that optimum pH for POD

from a new loquat fruit was (5.0pH). The lowest POD activity significant result was (0.52U/ml) at 5.0pH.

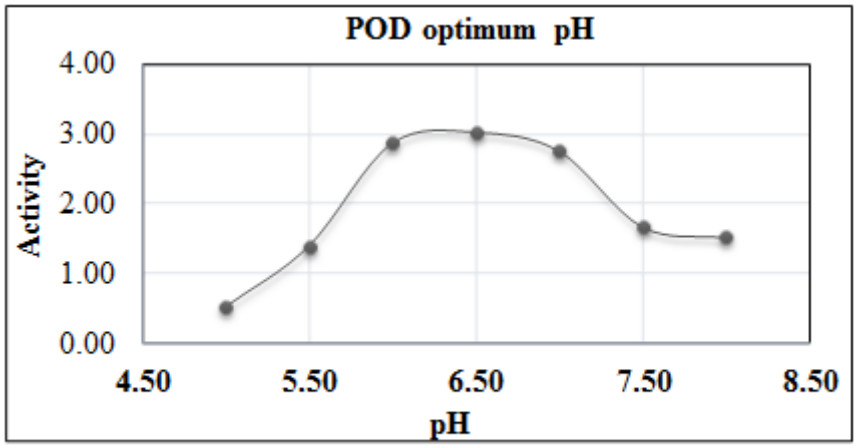

Fig (3) Effect of the reaction pH on purified POD from date Palm (cv. Barhee) callus.

Optimum temperature:

The purified POD with highest purification fold was tested at different temperature range (10 – 70^{o}C) to determine the optimum temperature for POD enzyme.

As shown in **Fig (4)**; the activity of POD from date palm (cv. Barhee) callus increased with temperature and reach to highest activity (3.33U/ml) at optimum temperature (40^{o}C). This obtained result was compatible with the result of **Altın *et al.* (2017)** and incompatible with the result of **Amiour *et al.* (2016)** on peroxidase

from Deglet Nour and Ghars date palm fruits where the optimum temperature was 35°C. In our experiment POD activity at temperature over 40°C was decreased gradually with the increase of temperature to reach the lowest one (0.06U) at 70°C.

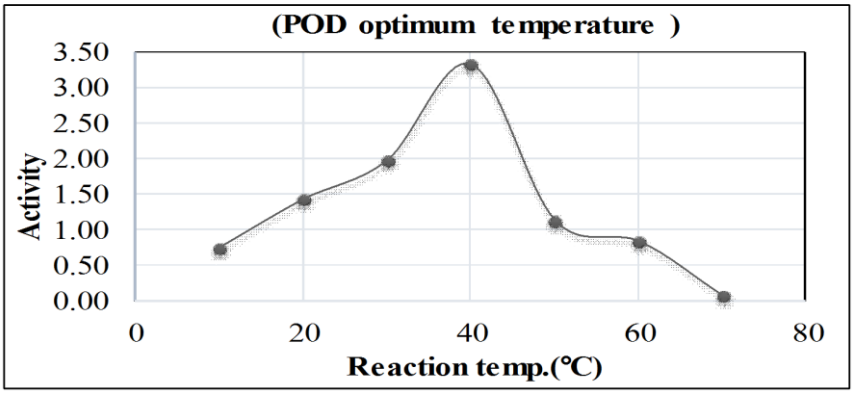

Fig (4) Effect of reaction temperature on purified POD from (*Phoenix dactylifera* L. cv Barhee) callus.

Substrate concentration and kinetic constants (K_m and V_{max}):

The effect of substrate (guaiacol and H_2O_2) concentration on partial purified POD was accomplished to determine the kinetic constants K_m (Michaelis constant) and V_{max} (the maximum reaction velocity) using construction of Woolf plot.

Data were plotted graphically to calculate kinetic constants (K_m and V_{max}) for the purified POD with guaiacol **Fig (5)** or H_2O_2 **Fig (6)** Calculated K_m and V_{max} recorded 0.4mM/reaction and 0.87U.min^{-1} respectively with constant level of H_2O_2. Meanwhile, calculated K_m and V_{max} for the purified POD with different H_2O_2 concentrations were 0.24mM/reaction and 0.44U.min^{-1}, respectively under constant level of guaiacol. The obtained result did not agree with the result obtained by **Tao *et al.* (2018)** for POD from jackfruit who reported K_m and V_{max} 3.7mM and 5.4 ΔOD.min^{-1} for guaiacol and 0.2 mM and 5.4 ΔOD.min^{-1} for H_2O_2 substrates, respectively.

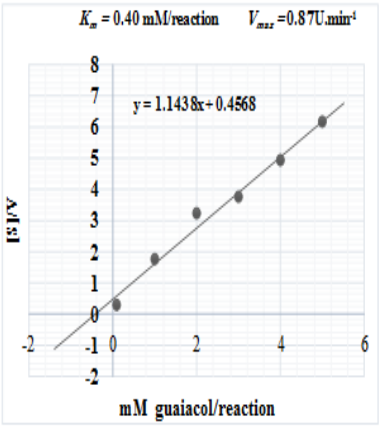

Fig (5) Woolf plot purified POD from (*Phoenix dactylifera* L. cv Barhee) callus using guaiacol.

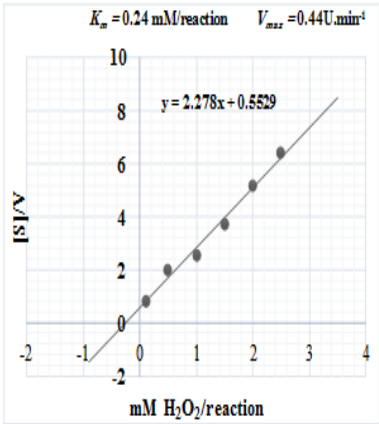

Fig (6) Woolf plot purified POD from (*Phoenix dactylifera* L. cv Barhee) callus using H_2O_2.

CONCLUSION

Peroxidase (POD) from (*Phoenix dactylifera* L. cv Barhee) showed higher activity during callus stage. The higher activity of POD may activate browning process because induction of peroxidase is related to oxidation of phenolic compounds. Purified POD had molecular weight of 43 kDa and showed activity range too close to standard pH for tissue culture media (pH5.7–5.8). Also, the optimum temperature of purified POD was found in the temperature range from 30 to 40^{o}C. The K_m and V_{max} were 0.4mM/reaction and 0.87U.min^{-1} for guaiacol whereas for H_2O_2 were 0.24mM/reaction and 0.44U.min^{-1}, respectively. From both data of optimum conditions and kinetics of POD enzyme obtained from Barhee callus, it could control POD activity and hence callus browning especially that there are a lot of callus source could be lost during this stage without any development.

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تنقية وتوصيف البيروكسيديز في الكالس من نخيل البلح البرحي

محمود علي مصطفى[1]، محمد عبد الرسول عبد الباقي[1]، محمد سعيد عباس[2]، أميرة شوقي سليمان[2]

[1]المعمل المركزي لأبحاث وتطوير نخيل البلخ، مركز البحوث الزراعية ،الجيزة

[2] قسم الموارد الطبيعية ، معهد البحوث والدراسات الإفريقية ، جامعة القاهرة ، الجيزة

البيروكسيديز واحد من المنظومة الإنزيمية الدفاعية ضد انواع الاكسجين النشط في نخيل البلح وخصوصا اثناء مرحلة الكالس. تهدف هذه الدراسة الي أستخلاص البيروكسيديز من الكالس في نخيل البلح البرحي من اجل التوصيف الكيميائي .وصل نشاط البيروكسيديز في المستخلص الخام من الكالس (161.14 وحدة). البيروكسيديز المنقى جزئيا بواسطة الايثانول (30%) تضاعفت فيه التنقية الي (7.14 ضعف) بينما ازدادت بواسطة سيفاديكس جي 100 الي (61.77 ضعف) . الوزن الجزيئي للبيروكسيديز المنقى كان (43 ك .د) بينما كان الاس الهيدروجيني الأمثل ودرجة الحرارة المثلى (6.5 و 40 سيليزوس) علي التوالي .وكانت الثوابت الحركية ثابت ميكاليس والسرعة القصوى للإنزيم للجوايكول 0.4ملمولر/تفاعل، 0.87 وحدة.دقيقة$^{-1}$ علي التوالي،ولفوق اكسيد الهيدروجين 0.24ملمولر/تفاعل، 0.44 وحدة.دقيقة$^{-1}$. ظاهرة التلون البني في الكالس للبرحي يمكن ان ترجع الي زيادة نشاط البيروكسيديز والتي يصاحبها دائما اكسدة للمركبات الفينولية. تحديد الظروف المثلى للبيروكسيديز من الكالس في نخيل البلح البرحي تمكن من التحكم في ظاهرة التلون البني بالاضافة الي ان يكون مصدر طبيعي لانتاج الانزيم يمكن استخدامه في تطبيقات اخرى.