Original Article

Partial purification and biochemical characterization of peroxidase from rosemary (*Rosmarinus officinalis* L.) leaves

Zahra Aghelan, Seyed Ziyaedin Samsam Shariat

Department of Clinical Biochemistry, School of Pharmacy and Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: In this study, it is aimed to purify POD from leaves of *Rosmarinus officinalis* L. and determine its some biochemical properties. PODs are a group of oxidoreductase enzymes that catalyze the oxidation of a wide variety of phenolic compounds in the presence of hydrogen peroxide as an electron acceptor.

Materials and Methods: In this investigation, POD was purified 9.3-fold with a yield of 32.1% from the leaves of Rosemary by ammonium sulfate precipitation and ion-exchange chromatography. The enzyme biochemical properties, including the effect of pH, temperature and ionic strength were investigated with guaiacol as an electron donor. For substrate specificity investigation of the enzyme, Michaelis constant and the maximum velocity of an enzymatic reaction values for substrates guaiacol and 3,3′, 5,5′-TetraMethyle-Benzidine were calculated from the Lineweaver–Burk graphs.

Results: The POD optimum pH and temperature were 6.0 and 40°C. The POD activity was maximal at 0.3 M of sodium phosphate buffer concentration (pH 6.0). Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for molecular weight (M_w) determination and M_w of the enzyme was found to be 33 kDa. To investigate the homogeneity of the POD, native-PAGE was done and a single band was observed.

Conclusion: The stability against high temperature and extreme pH demonstrated that the enzyme could be a potential POD source for various applications in the medicine, chemical and food industries.

Key Words: Ion-exchange chromatography, peroxidase purification, *Rosmarinus officinalis* L.

Address for correspondence:
Dr. Seyed Ziyaedin Samsam Shariat, Department of Clinical Biochemistry, Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: samsam@pharm.mui.ac.ir

Received: 28.12.2014, **Accepted:** 17.02.2015

INTRODUCTION

Peroxidases (PODs) (donor: H_2O_2 oxidoreductase, EC: 1.11.1.7) belong to a large family of enzymes which contain a ferriprotoporphyrin IX prosthetic group[1] and catalyze the oxidation of a wide variety of phenolic compounds such as guaiacol, pyrogallol, acid chlorogenic, catechin and catechol in the presence of hydrogen peroxide or organic hydroperoxides.[2] In 1936 the enzyme was first found in the fig tree that was isolated and characterized from horseradish in 1941.[3] Multiple forms of POD widely distributed in most living organisms including plants, microbes and animal tissues.[4] Plant POD are mainly located in tonoplast and plasmalema, inside and outside the cell wall[5] and participate in diverse physiological functions such as lignification process[6] and in the

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.161586

How to cite this article: Aghelan Z, Shariat SS. Partial purification and biochemical characterization of peroxidase from rosemary (*Rosmarinus officinalis* L.) leaves. Adv Biomed Res 2015;4:159.

mechanism of defense in physiocally damaged or infected tissues.[7] The POD superfamily can be divided into three classes according to their origin, amino acid homology and metal-binding capability.[8] Class III POD are found in several large family of plants[9] and are monomeric glycoproteins containing four conserved disulfide bridges which are activated in the presence of calcium ions.[10] PODs from different sources are widely used in the clinical, biochemical, biotechnological and industrial fields.[11] These enzymes are considered as a reagent for organic synthesis and biotransformation as well as coupled enzyme assays, chemiluminescent assays, immunoassays and treatment of waste waters.[12] Despite the variety of plant peroxidase sources, there is no previous study on peroxidase from *Rosmarinus officinalis* L. leaves. *R. officinalis* L., commonly known as rosemary, is a woody, perennial herb with fragrant, evergreen needle-like leaves, native to the Mediterranean region which now grows widely in other parts of the world. The plant has been shown to have antioxidant properties.[13] Thus, rosemary is an important plant having nutritional and medicinal value. In this present study, we partially purified and determined the biochemical characterization of POD from the leaves of Rosemary as a new source of POD for different applications.

MATERIALS AND METHODS

Plant material

Fresh leaves of Rosemary were collected from the campus of Isfahan University of Medical Sciences. Then, they were transported to the laboratory, washed and used for preparation of crude extract.

Preparation of plant extract

Thirty gram from the Rosemary leaves were ground with a mortar and homogenized in phosphate buffer, pH 6.0 (0.3 M) containing polyvinylpyrrolidone (0.05 %w/v) using ice cold blender. The homogenate was centrifuged at 15000×g for 60 min at 4°C using Sigma 3K30 centrifuge. The supernatant was collected.[14]

Purification of the anionic peroxidase

The crude extract was subjected to 10–90% ammonium sulphate saturation. Then the mixture was centrifuged at 15000×g for 60 min at 4°C using Sigma 3K30 centrifuge and the precipitate was suspended in the minimum volume of phosphate buffer (pH 6.0, 0.3 M). Afterward, it was dialyzed for 12 h at 4°C against 1 L the same buffer.[14] The dialyzed enzyme sample was loaded onto the diethyl amino ethyl (DEAE)-cellulose column (2.5 cm × 30 cm) pre-equilibrated with 0.02 M phosphate buffer (pH 8.0) at 4°C and eluted with a 0.0 – 0.5 M Nacl linear gradients in the same buffer at a flow rate of 0.7 ml/min. The eluted fractions of 3 ml were collected and each activity and absorbance was separately measured at 470 nm and 280 nm, respectively.[8] Active fractions were pooled and kept at 4°C until use.

Protein electrophoresis

To check the purity of the obtained peroxidase, native- polyacrylamide gel electrophoresis (PAGE) was performed by making 10% resolving gel with 3% stacking gel according to laemmlis procedure.[15] Enzyme samples were loaded onto the wells of the stacking gel and electrophoresis was run at 120 constant voltage mode using Tris-glycine running buffer, pH 8.3. After running, gel was incubated in 45 mM guaiacol and 22.5 mM H_2O_2 in 0.3 M phosphate buffer (pH 6.0) at 37°C until appearance of the enzyme bands.[14]

Molecular weight determination

Sodium dodecyl sulphate (SDS)-PAGE was used for determination of molecular weight (M_W) of POD from rosemary. The sample was boiled in the presence of SDS and 2-mercaptoethanol and separated in a 10% gel according to the method of laemmli.[15] The standard proteins used for SDS-PAGE were β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), endonuclease (25 kDa), β-lactogolobin (18.4 kDa) and lysozyme (14.4 kDa). The M_W of the purified POD was determined by a calibration curve (log molecular weights of the standards vs. retention factor values).

Peroxidase activity assay

The POD activity assay was performed with the help of koksals protocol[4] with slight modifications, using guaiacol as substrate by measurement of the absorbance at 470 nm of 3,3′-Dimethoxy-4,4′-biphenoquinone (ε = 6.39/mM/cm) at room temperature. The reaction mixture (total volume, 1 ml) consisted of sodium phosphate buffer, pH 6.0 (0.3 M), guaiacol (45 mM), H_2O_2 (22.5 mM) and 25 µl of enzyme solution. One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 mmol of 3,3′-Dimethoxy-4,4′-biphenoquinone per min.[16]

Qualitative and quantitative protein determination

During the purification process, the measurement of quantitative protein amount was carried out spectrophotometrically at 595 nm according to Bradfords method,[17] with bovine serum albumin as standard. Qualitative protein measurement was performed at 280 nm on the eluates obtained.[18]

CHARACTERIZATION OF PURIFIED PEROXIDASE

Effect of temperature

The effect of temperature on the purified POD activity was determined by incubating the assay

system at different temperatures ranges from 10°C to 80°C for 5 min and POD activity was measured spectrophotometrically. The percentage POD activity was calculated by comparison with unheated enzyme. Thermal stability of the POD was determined by incubating purified enzyme solution in a test tube at 40°C, 50°C and 60°C for fixed time intervals (10,20,30,40,50,60 min).[14]

Effect of PH

The effect of pH on the POD activity was investigated using wide range of pH 3.0–9.0 in 0.3 M buffers (sodium acetate, pH 3.0–5.0; sodium phosphate, pH 6.0–7.0; Tris-HCL, pH 8.0–9.0) at fixed concentration of guaiacol and H_2O_2.[19]

Effect of ionic strength

The effect of ionic strength on the purified POD activity was determined by measuring the activity of the enzyme with different concentrations of sodium phosphate buffer, pH 6.0 (0.05–2 M) at a fixed concentration of guaiacol and H_2O_2.

Substrate specificity

Under optimal conditions, the effect of different substrates on the activity of POD from rosemary was investigated by determining the activity with five different concentrations of guaiacol (12–75 mM) and 3,3′,5,5.-Tetramethyl-benzidine (TMB) (2–10 mM) at a fixed H_2O_2 concentration and five different concentrations of H_2O_2 (12–70 mM) at a fixed concentration of guaiacol. Michaelis constant (K_m) and maximum velocity of an enzymatic reaction (V_{max}) values were calculated for POD reactions with each of this substrates by the Lineweaver–Burk graph.[20]

Statistical analysis

Data were subjected to ANOVA (one-way variance analysis) using statistical software SPSS 17. General Linear Model procedure was performed to examine the effects of temperature, pH and interaction of each factor with time on the stability of enzyme. The means were presented for averages of experiments that were repeated at least three times. Means values were compared by *post hoc* Tukey test. The term significant indicates differences for which $P < 0.05$.

RESULTS

Peroxidase from the leaves of rosemary was isolated and purified through two steps of ammonium sulphate fractionation and DEAE-cellulose chromatography. The fraction obtained in 80–90% saturation range showed the maximum activity. As the result of this step, the POD was purified to 3.0-fold with a yield of 66.2%. The enzyme then loaded on a DEAE-cellulose column and bound proteins were eluted with a linear gradient of 0.0–0.5 M Nacl in 0.02 M phosphate buffer (pH 8.0) at a flow rate of 0.7 ml/min [Figure 1]. The result of the purification of POD from the rosemary leaves is shown in Table 1. As can be seen in figure 2, this enzyme exhibited a single band on native-PAGE and SDS-PAGE that was suggestive of the homogeneity of the purified POD. The M_W of the purified POD was estimated as 33 kDa according to the M_W markers on SDS-PAGE [Figure 2]. The effect of temperature on the purified POD was studied at different temperatures from 10°C to 80°C. The enzyme was heat stable over a wide range of temperatures (0–70°C). The optimum temperature of the POD was found to be 40°C for guaiacol [Figure 3]. In addition, this enzyme can be stable at 60°C for 10 min [Figure 4]. The effect of pH on the enzyme was determined by measuring the activity at different pH range of 2.0–9.0 [Figure 5]. The enzyme showed the highest activity in pH 6.0. The effect of ionic strength on the POD activity was determined at a fixed concentration of guaiacol and H_2O_2 by different concentrations of sodium phosphate buffer, pH 6.0 (0.05–2 M). The maximum activity was observed in 0.3 M of phosphate buffer concentration at pH 6.0 [Figure 6]. K_m and V_{max} values were calculated for guaiacol, TMB and H_2O_2 substrates from Lineweaver-Burk graphs. The K_m values of the POD for guaiacol, TMB and H_2O_2 substrates were 28.8, 1.06 and 8.5 mM, respectively. Indeed, V_{max} values were 0.312, 0.194 and 1.15 mM/min for above mentioned substrates, respectively [Figure 7].

DISCUSSION

Peroxidase enzyme from the *R. officinalis* L. leaves was extracted and purified by two steps

Table 1: Steps of purification of peroxidase isolated from rosemary leaves

Purification steps	Total volume (ml)	Total enzyme activity (unit*)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	100	4081.3	84	48.5	100	1.0
$(NH_4)_2SO_4$ precipitatin	25	2704.2	18.5	146.1	66.2	3.0
DEAE-cellulose chromatography	15	1310.7	2.9	451.9	32.1	9.3

*One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 mmol of 3,3'-dimethoxy-4,4'-biphenoquinone formed per minute at room temperature

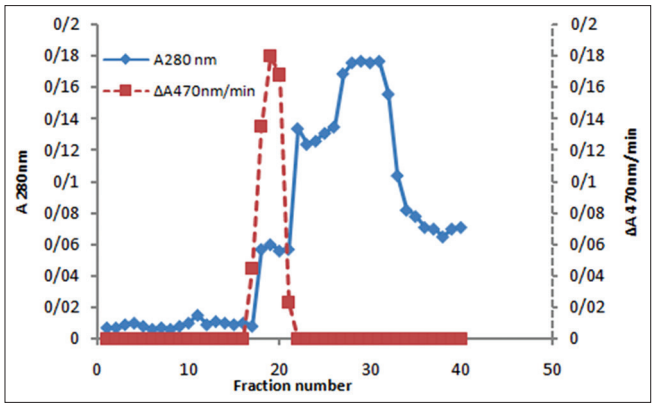

Figure 1: Diethyl amino ethyl (DEAE)-cellulose ion exchange chromatography of peroxidase from the leaves of rosemary: The dialyzed enzyme sample was loaded onto the DEAE-cellulose column, preequilibrated with 0.02 M phosphate buffer (pH 8.0) at 4°C and eluted with 0.0–0.5 M Nacl linear gradients in the same buffer at a flow rate of 0.7 ml/min. The eluted fractions of 3 ml were collected

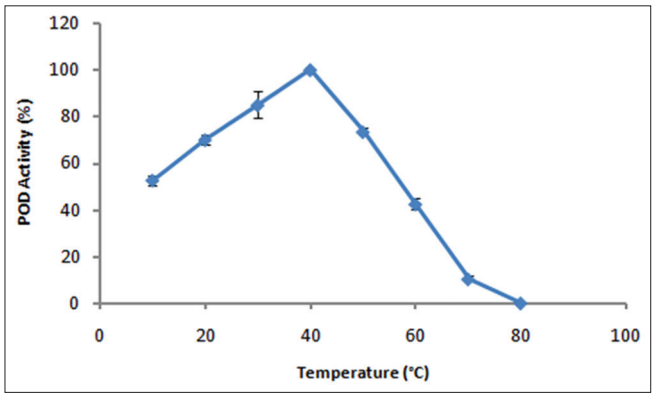

Figure 3: The effect of temperature, 10–80°C, on the peroxidase (POD) activity from rosemary leaves. The optimum temperature of the POD was found to be 40°C for guaiacol. Data are shown as a mean of the activity ± standard deviation of $n = 3$ experiments

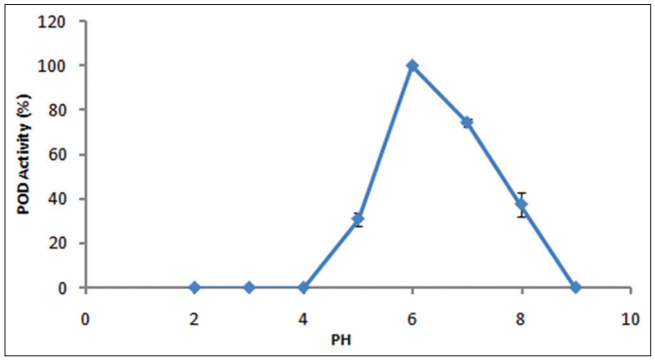

Figure 5: The effect of pH on the activity of the purified peroxidase from rosemary leaves. The peroxidase activity was determined using a wide range of pH 3–9 in 0.3 M buffers (sodium acetate, pH 3.0–5.0; sodium phosphate, pH 6–7; Tris-HCL, pH 8–9). Data are shown as a mean of the activity ± standard deviation of $n = 3$ experiments

of ammonium sulphate fractionation and ion exchange chromatography. The POD was purified to 9.3-fold with a yield of 32.1% and specific activity

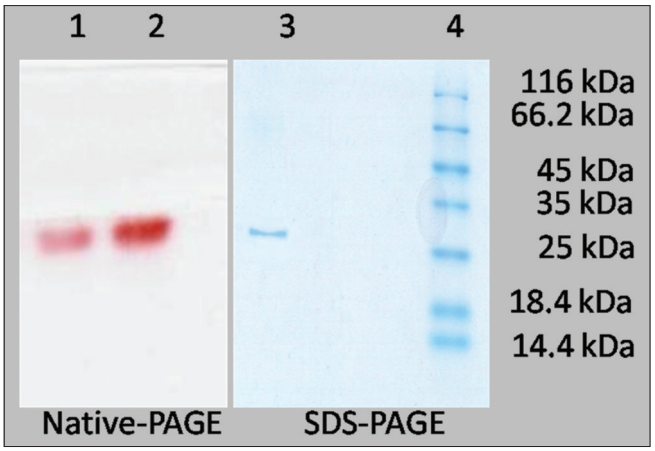

Figure 2: Electrophoresis of the purified peroxidase (POD) (25 μl) from the rosemary leaves: (1) The purified POD in the presence 2-mercaptoethanol, (2) purified POD without 2-mercaptoethanol; (3) purified POD by diethyl amino ethyl-cellulose ion exchange chromatography, (4) standard proteins β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), endonuclease (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa)

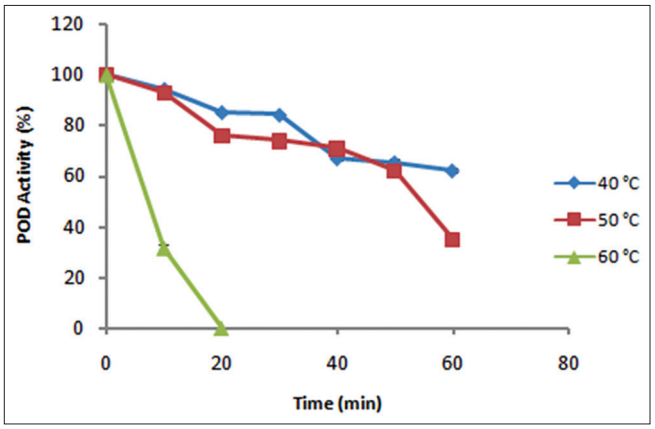

Figure 4: Thermal stability of the purified peroxidase from rosemary leaves. The enzyme was incubated at the 40°C, 50°C and 60°C for fixed time intervals (10, 20, 30, 40, 50 and 60). Data are shown as a mean of the activity ± standard deviation of $n = 3$ experiments

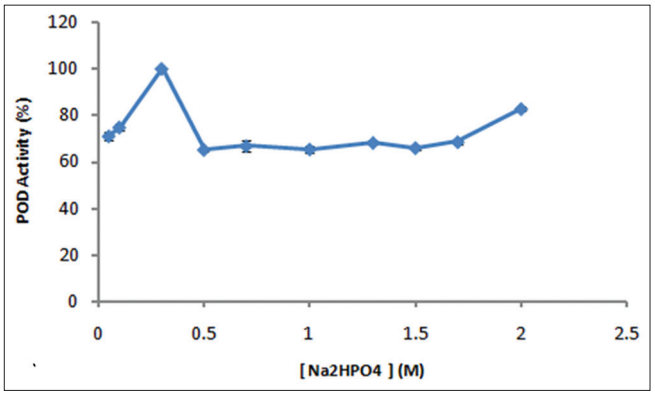

Figure 6: The effect of ionic strength on the activity of the purified peroxidase from rosemary leaves. The activity of the enzyme was determined with different concentrations of sodium phosphate buffer, pH 6 (0.05–2 M) at a fixed concentration of guaiacol and H_2O_2. Data are shown as a mean of the activity ± standard deviation of $n = 3$ experiments

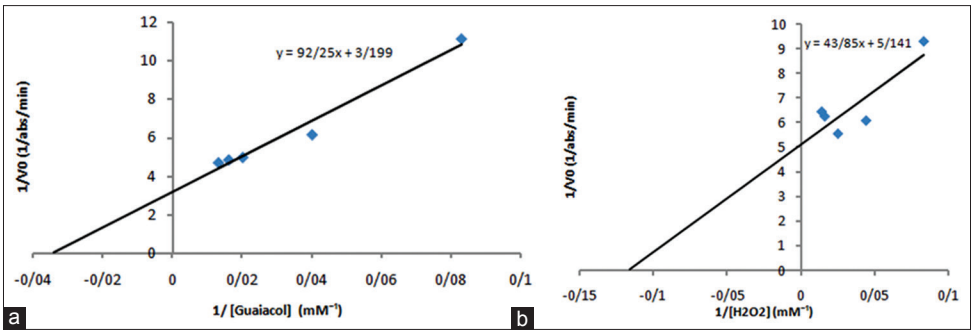

Figure 7: Lineweaver–Burk graph of purified peroxidase (POD) from rosemary leaves. (a) The POD activity was measured in the presence of increasing guaiacol concentrations at a fixed H_2O_2 concentration. The K_m and V_m values of the POD for guaiacol were 28.8 mM and 0.312 mM/min, respectively. (b) The POD activity was measured in the presence of increasing H_2O_2 concentrations at a fixed guaiacol concentration. The K_m and V_m values of the POD for H_2O_2 were 8.5 mM and 1.15 mM/min, respectively. Data are shown as a mean of activity of three separate experiments

of 451.9 unit/mg. Peroxidase from the leaves of rosemary showed a single band on native-PAGE and SDS-PAGE, demonstrating that the purified enzyme is composed of a single polypeptide chain. The effect of β-mercaptoethanol on this POD activity indicated that at least one disulfide bond within the structure was important for its activity. The M_w of this enzyme was estimated as 33 kDa by SDS-PAGE while the M_w of different POD have been found between 32 and 45 kDa[21] such as Turnip (C_3) with 34 and 38 kDa,[22] Lettus with 35 kDa,[23] Horseradish root with 40 kDa[24] and Chick pea with 39 kDa.[25] The optimal pH value of rosemary POD was found to be 6.0. A similar optimum pH for the purified POD has been reported from Turkish black radish,[26] *Copaifera langsdorffii*,[27] *Moringa oleifera*[28] and Broccoli.[2] This peroxidase did not show activity at acidic pH <4 while at pH 8.0, 37.6% activity was retained for the purified POD. Indeed, the isolated peroxidase from rosemary was stable at a pH range from 4.0-8.0. At low PH loss of activity is due to heme disruption from the enzyme.[29] Because the POD can be used for the various industrial applications, the knowledge of the optimum temperature on POD activity is an essential step towards more efficient applications of this enzyme.[30] The maximum activity of this obtained peroxidase was observed at 40°C. A wide variability in the optimum temperature has been reported for POD from various sources. The soluble POD of several plant tissues exhibited optimum temperature between 30°C and 60°C.[4,14,22,31,32] The plant POD are glycosylated proteins and regarded as one of the most heat-stable enzymes (23). Because POD catalyze the oxidation of several substrates as an electron donor in the presence of hydrogen peroxide,[33] we calculated K_m and V_{max} values using the Linweaver–Burk graph for guaiacol, TMB and H_2O_2 for substrate specificity determination. The lowest Km with TMB and the highest Vmax/Km shows that the enzyme has a greater tendency toward hydrogen peroxide in the presence of TMB. The concentration of H_2O_2 is an important factor in measuring the POD activity and when the H_2O_2 concentration is less than the concentration of the guaiacol, the reaction velocity is linearly correlated with the concentration of H_2O_2 and with increasing concentration of 40 mM of H_2O_2, POD activity is reduced. As a Conclusion, this POD showed high stability at a wide range of pH and temperature and is an inexpensive and easily available source of POD. Thus, our study introduces a new source of POD that can be used as an industrial enzyme with applications in the medicine, chemical and food industries.

ACKNOWLEDGMENTS

This work was supported by the Research Council of Isfahan University of Medical Sciences.

REFERENCES

1. Nouren S, Bhatti HN, Bhatti IA, Asgher M. Kinetic and thermal characterization of peroxidase from peels of *Citrus reticulate* var. kinnow. J Anim Plant Sci 2013;23:430-5.
2. Thongsook T, Barrett DM. Purification and partial characterization of broccoli (*Brassica oleracea* Var. Italica) peroxidases. J Agric Food Chem 2005;53:3206-14.
3. O'donnell J, Huystee RV. Peanut peroxidase and the anionic component. Can J Bot 1992;70:1131-3.
4. Koksal E. Peroxidase from leaves of spinach (*Spinacia oleracea*): Partial purification and some biochemical properties. Int J Pharm 2011;7:135-9.
5. Vitali A, Botta B, Delle Monache G, Zappitelli S, Ricciardi P, Melino S, *et al.* Purification and partial characterization of a peroxidase from plant cell cultures of Cassia didymobotrya and biotransformation studies. Biochem J 1998;331 (Pt 2):513-9.
6. Wakamatsu K, Takahama U. Changes in peroxidase activity and in peroxidase isozymes in carrot callus. Physiol Plant 1993;88:167-71.
7. Biles C, Martyn R. Peroxidase, polyphenoloxidase, and shikimate dehydrogenase isozymes in relation to tissue type, maturity and pathogen induction of watermelon seedlings. Plant Physiol Biochim 1993;31:499-506.
8. Chanwun T, Muhamad N, Chirapongsatonkul N, Churngchow N. Hevea brasiliensis cell suspension peroxidase: Purification, characterization and application for dye decolorization. AMB Express 2013;3:14.
9. Cosio C, Dunand C. Specific functions of individual class III peroxidase genes. J Exp Bot 2009;60:391-408.
10. Schuller DJ, Ban N, Huystee RB, McPherson A, Poulos TL. The crystal structure of peanut peroxidase. Structure 1996;4:311-21.

11. Srinivas N, Barhate R, Raghavarao K. Aqueous two-phase extraction in combination with ultrafiltration for downstream processing of Ipomoea peroxidase. J Food Eng 2002;54:1-6.
12. Veitch NC. Horseradish peroxidase: A modern view of a classic enzyme. Phytochemistry 2004;65:249-59.
13. Angioni A, Barra A, Cereti E, Barile D, Coïsson JD, Arlorio M, *et al.* chemical composition, plant genetic differences, antimicrobial and antifungal activity investigation of the essential oil of *Rosmarinus officinalis* L. J Agric Food Chem 2004;52:3530-5.
14. Köksal E, Gülçin I. Purification and characterization of peroxidase from cauliflower (*Brassica oleracea* L. var. botrytis) buds. Protein Pept Lett 2008;15:320-6.
15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
16. Doerge DR, Divi RL, Churchwell MI. Identification of the colored guaiacol oxidation product produced by peroxidases. Anal Biochem 1997;250:10-7.
17. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
18. Soyut H, Beydemir S. Purification and some kinetic properties of carbonic anhydrase from rainbow trout (*Oncorhynchus mykiss*) liver and metal inhibition. Protein Pept Lett 2008;15:528-35.
19. Sakharov IY, Castillo J, Areza JC, Galaev IY. Purification and stability of peroxidase of African oil palm *Elaies guineensis*. Bioseparation 2000;9:125-32.
20. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc 1934;56:658-66.
21. Rompel A, Albers M, Naseri JI, Gerdemann C, Büldt-Karentzopoulos K, Jasper B, *et al.* Purification, cloning and characterization of a novel peroxidase isozyme from sweet potatoes (Ipomoea batatas). Biochim Biophys Acta 2007;1774:1422-30.
22. Saboora A, Parsiavash L, Moosavi-Nejad Z. Purification and kinetic properties of guaiacol peroxidase in turnip (*Brassica napus* var. okapi) root during different growth stages. Prog Biol Sci 2012;2:76-86.
23. Hu Y, Wu J, Luo P, Mo Y. Purification and partial characterization of peroxidase from lettuce stems. Afr J Biotech 2012;11:2752-8.
24. Lavery CB, Macinnis MC, Macdonald MJ, Williams JB, Spencer CA, Burke AA, *et al.* Purification of peroxidase from Horseradish (*Armoracia rusticana*) roots. J Agric Food Chem 2010;58:8471-6.
25. Bhatti HN, Najma A, Asgher M, Hanif MA, Zia MA. Purification and thermal characterization of a novel peroxidase from a local chick pea cultivar. Protein Pept Lett 2006;13:799-804.
26. Şişecioğlu M, Gülçin İ, Çankaya M, Atasever A, Şehitoğlu MH, Kaya HB, *et al*. Purification and characterization of peroxidase from Turkish black radish (*Raphanus sativus* L). J Med Plant Res 2010;4:1187-96.
27. Maciel HP, Gouvêa CM, Toyama M, Smolka M, Marangoni S, Pastore GM. Extraction, purification and biochemical characterization of a peroxidase from *Copaifera langsdorffii* leaves. Quím Nova 2007;30:1067-71.
28. Khatun S, Ashraduzzaman M, Karim M, Pervin F, Absar N, Rosma A. Purification and characterization of peroxidase from *Moringa Oleifera* L. Leaves. Bioresources 2012;7:3237-51.
29. Adams CD, Gorg S. Effect of pH and gas-phase ozone concentration on the decolorization of common textile dyes. J Environ Eng 2002;128:293-8.
30. Sakharov IY. Palm tree peroxidases. Biochemistry (Mosc) 2004;69:823-9.
31. Loukili AE, Limam F, Ayadi A, Boyer N, Ouelhazi L. Purification and characterization of a neutral peroxidase induced by rubbing tomato internodes. Physiol Plant 1999;105:24-31.
32. Pandey VP, Singh S, Singh R, Dwivedi UN. Purification and characterization of peroxidase from papaya (*Carica* papaya) fruit. Appl Biochem Biotechnol 2012;167:367-76.
33. Yemenicioglu A, Özkan M, Cemeroglu B. Partial purification and thermal characterization of peroxidase from okra (*Hibiscus esculentum*). J Agric Food Chem 1998;46:4158-63.

Source of Support: Nil, **Conflict of Interest:** None declared.