

Peroxidase from *Coleus Forskohlii*: Purification and Biochemical Characterization

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Abstract

In this study, a peroxidase from new source was purified using ion exchange and gel filtration techniques. The recovery for peroxidase activity was 19% with 11-fold purification and specific activity of 749 unit/mg protein. Purified peroxidase demonstrated a molecular mass of 39 kDa using gel filtration and was confirmed as a single band on SDS-PAGE. The purified peroxidase revealed a broad optimum pH activity at 6.0-6.5 and 50°C temperature. The kinetic parameters for purified peroxidase toward H₂O₂ and guaiacol as substrates were found to be $K_m = 3.355, 5.395 \text{ mM}$, $K_{cat} = 99.52, 79.56 \text{ s}^{-1}$ and $V_{max} = 1.531, 1.242 \text{ } \mu\text{mole ml}^{-1} \text{ min}^{-1}$, respectively. The catalytic efficiency (k_{cat}/K_m) of the purified peroxidase was 14.75 and 29.66 $\text{s}^{-1} \text{ mM}^{-1}$ for guaiacol and H₂O₂, respectively. Peroxidase activity was observed to be enhanced by Cu²⁺, Co²⁺, Ni²⁺ and inhibited in the presence of Sn²⁺, Al³⁺, Hg²⁺, NaN₃, EDTA and urea. Characterization showed that peroxidase purified from *C. forskohlii* has the ability to be used for food industrial applications.

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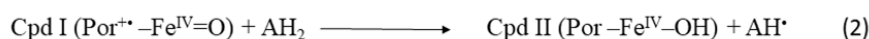
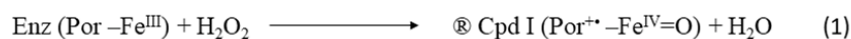
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Introduction

Medicinal plants have been used for a long time since the advent of human civilisation [1]. It is an old practice to use these plants for their medicinal benefits. Their roots in the world's oldest cultures remain well-preserved for many decades [2]. *C. forskohlii* is a persistent medicinal plant of the Lamiaceae family and has been distributed throughout the world, including Saudi Arabia, China, India, Africa and Pakistan [3,4]. An extract of this plant was used in Ayurvedic medicine for centuries to treat different health problems [5]. Forskolin, the main diterpene component of this plant, is used as an antioxidant compound for various disorders [6]. The antioxidants, including enzymatic antioxidants, can help the body defend itself from

various kinds of reactive oxygen damage caused by a variety of conditions. [4]. Peroxidase (E.C.1.11.1.7) is belong to the oxidoreductases family, normally found in plants [7,8], and responsible for the browning process [9]. Peroxidase can facilitate plant darkening, although the existence of electron-acceptor compounds such as lipid peroxides, hydrogen peroxide, and superoxide radicals are limits it [10,11]. The catalytic reaction of peroxidases takes place in three different steps. The initial phase includes oxidation of peroxidase to create an unstable intermediate compound called (Cpd I) (1). In the second phase, Cpd I is reduced to Cpd II and to a free radical by an appropriate electron donor (2). Then the second substrate further reduces Cpd II to recover the resting state of the enzyme and another radical one (3) [12].



Specific physiological roles of peroxidases in plants include suberisation, lignification, wound healing, catabolism of auxins, porphyrin metabolism, hydrogen peroxide scavenging, organogenesis and senescence [13]. In the presence of hydrogen peroxide, they catalyze the oxidation of different phenolic and nonphenolic compounds [14]. Because of their diverse substrate specificity, availability, and high sensitivity, Plant peroxidase has been commonly used in several applications, including environmental protection, such as phenolic wastewater bioremediation, bio pulping, and clinical diagnosis, etc [14]. In this study, the enzymatic properties of peroxidase have been studied from a new source. peroxidase from *C. forskohlii* was purified using ion exchange and gel filtration chromatography. The purity of the enzyme was checked using SDS-PAGE. Also, the physio biochemical properties of the purified enzyme were achieved.

Material and Methods

Chemicals and Plant Materials

Guaiacol, Sephacryl S-200, CM-Sepharose were acquired from Sigma-Aldrich. All other chemicals used

were of analytical grade. *Coleus forskohlii* is readily available because of its ubiquitous growth in the wild. The sample was obtained from Khulais, Saudi Arabia, in Sep. 2019, situated at 39°21'50"E110°22'58°21.

C. forskohlii Peroxidase Purification

Preparation of the Plant Extract

Fifteen grams of *Coleus forskohlii* peel stem had been ground in a 0.02 M Tris-HCl buffer, pH 7.2. Filtered and centrifuged this crude extract for 15 min at 12,000 rpm and discarded the pellet.

Ion Exchange and Gel Filtration Chromatography

Briefly, CM-Sepharose filled the column, equilibrated with 0.02 M Tris-HCl buffer, pH 7.2. The homogenous enzyme was then loaded onto the column and eluted at a flow rate of 30 mL/h in the same buffer with a gradient of 0.0–0.3 M NaCl. Four peaks were pooled with protein fractions exhibiting peroxidase activity. The fraction with the highest activity were applied on a Sephacryl S-200 column equilibrated with 20 mM Tris-HCl buffer (pH 7.2). peroxidase was again

eluted at a flow rate of 30 mL/h and quantities of 3 ml have been collected.

Protein Estimation

Bradford method [15] used to quantify the protein using bovine serum albumin as standard.

Determination of Molecular Weight

According to Laemmli method [16], the pooled enzyme from gel filtration chromatography was added to SDS-PAGE to check the purity of the enzyme and assess its molecular weight.

Enzyme Assay

The peroxidase activity was determined using H₂O₂ and guaiacol as substrates [17]. The reaction mixture contained 20 μ L enzyme sample, 40 mM guaiacol, 8 mM H₂O₂, and 50 mM phosphate buffer (pH 7.0).

Physicochemical Characterization of the Enzyme

Effect of pH

In the range of pH 4.0–9.0, the peroxidase activity was measured using 50 mM of acetate buffer (pH 4.0–6.0) and Tris/HCl buffer, (pH 6.5–9.0). The stability at the different pH levels (pH 5.0–8.5) was established by calculating the residual activity in buffers with the same pH after incubating the enzyme for 24 h at 4 °C.

Effect of Temperature and Thermal Stability

The temperature effect on purified peroxidase activity was performed at temperatures between 30 and 80 °C. To obtain thermal stability, the enzyme was pre-incubated at the working temperature for 30 min before the addition of substrates and starting the reaction.

Kinetic Parameters

K_m, K_{cat} and V_{max} values of peroxidase were determined for hydrogen peroxide and guaiacol substrates. The kinetic parameter values were calculated from Line Weaver-Burk graphs.

Effect of Metal Ions and Inhibitors

The effect of different metals and inhibitors on enzyme activity has also been investigated. Salt additives of CuSO₄, CoSO₄, NiCl₂, AlCl₃, SnCl₂ and Hg (NO₃)₂ (2.0–10 mM) have been used in this study. Peroxidase was additionally incubated with several

compounds, such as sodium azide (NaN₃), EDTA (2.0–10 mM) and Urea (0.2–1 M). The enzyme was pre-incubated with metals and/or inhibitors and buffer for 30 min before adding the substrates. Relative activity, against the control, was estimated. Control of peroxidase activity (without addition of metals and/or inhibitors) was considered to be 100% activity.

Results and Discussion

Purification of C. forskohlii Peroxidase

Prior to use in any industrial process, biochemical characterization of an enzyme is necessary [18]. In this study, C. forskohlii peroxidase purification was optimized and its biochemical properties were determined. As can be seen in Fig.1, four major protein peaks (A280) and four C. forskohlii peroxidase peaks have been obtained using CM-sepharose column chromatography. The fraction (POD3) with highest peroxidase activity were collected and concentrated. The purity of C. forskohlii peroxidase (POD3) was 3.5-fold with a cumulative recovery of 24 % (Table 1). Finally, to obtain a homogeneous peroxidase, the enzyme was applied to Sephacryl S-200 column (Fig.2A). The C. forskohlii peroxidase (POD3A) was purified at a yield of 19% and 11-fold purification with specific activity of 742 unit/mg protein. The purified peroxidase provided a single protein band of 12% SDS-PAGE (Fig.2B), confirming its uniformity. Using a Sephacryl S-200 column, the molecular weight of purified peroxidase was estimated to be 39 kDa. Moreover, SDS-PAGE research was carried out to determine and check molecular weight and purified peroxidase uniformity. These findings are in agreement with all those peroxides produced from other plant sources, for example, broccoli (48 kDa) [19], Commiphora gileadensis (40) [8], pearl millet grains (31 kDa) [20]. The results obtained from the effect of pH on the C. forskohlii peroxidase showed that the enzyme has a broad optimum pH from 6.0 to 6.5 (Fig.3a). Peroxidases from most plants are particularly active in pHs 4.5–7, but some peroxidases display a lower pH maximum activity (pH 3.8) [21]. To assess the effect of pH stability on C. forskohlii peroxidase, the purified enzyme was incubated at various pH ranging from 5.0 to 8.5 for 24 h at 4 °C (Fig. 3b). As seen in this figure, C. forskohlii peroxidase activity at pH ranging from 5.5 to

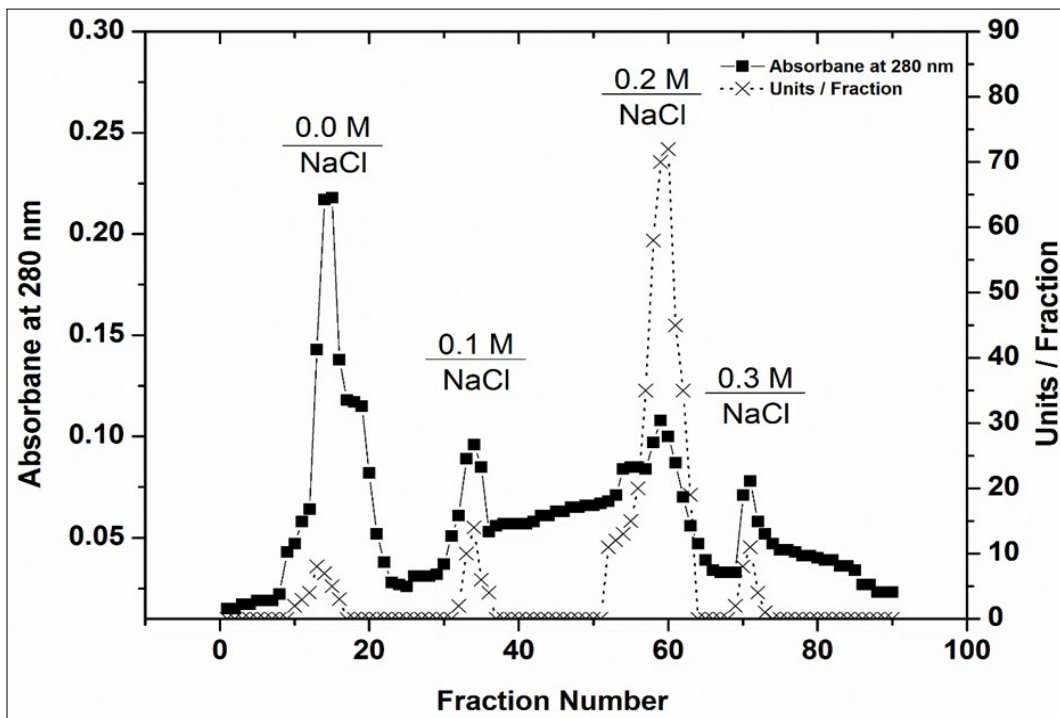


Figure 1. A typical elution profile for the chromatography of peroxidase using a CM-Sepharose column.

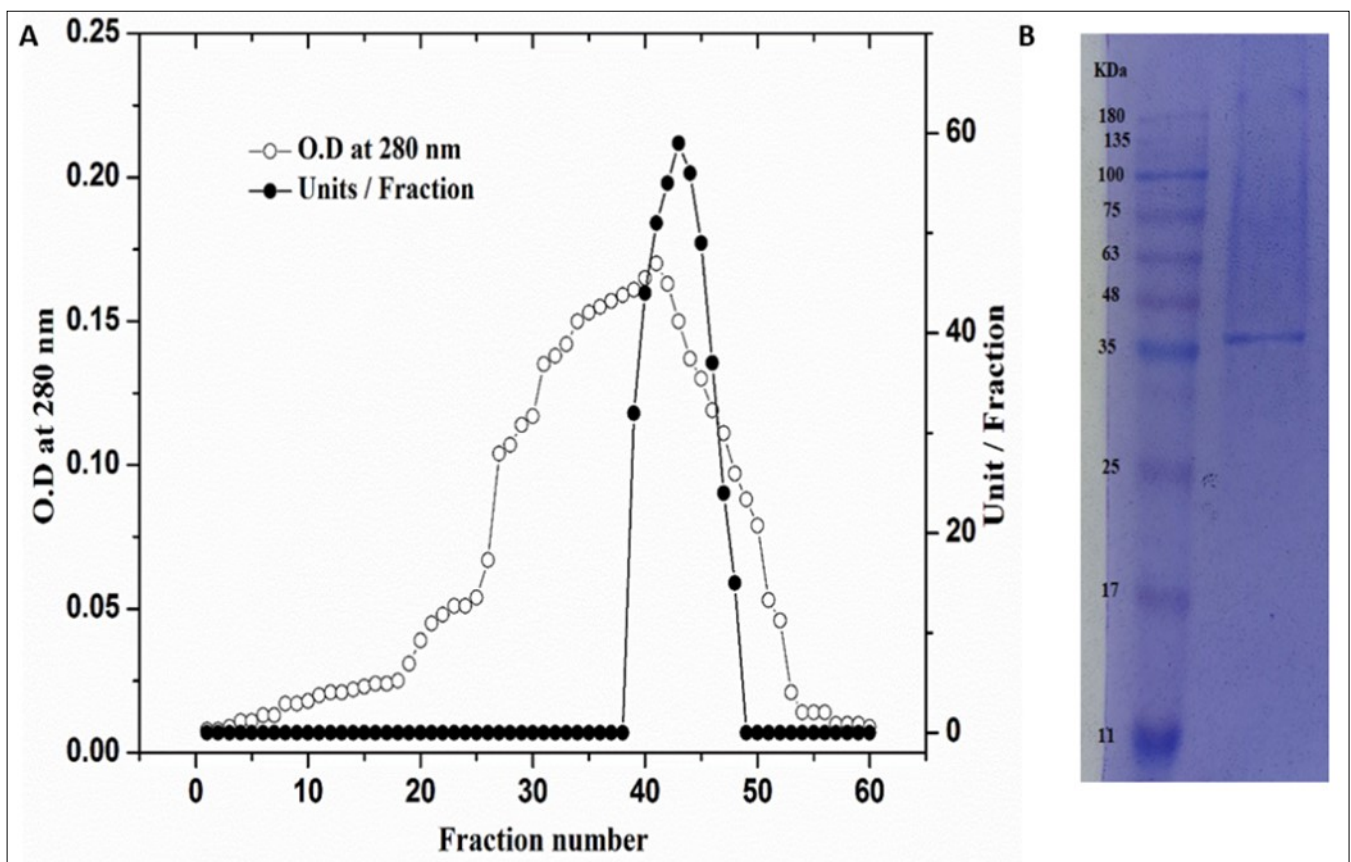


Figure 2. Gel filtration of POD3 CM-Sepharose fractions using a Sephacryl S-200 column (A), SDS-PAGE for homogeneity and molecular weight determination of peroxidase (B).

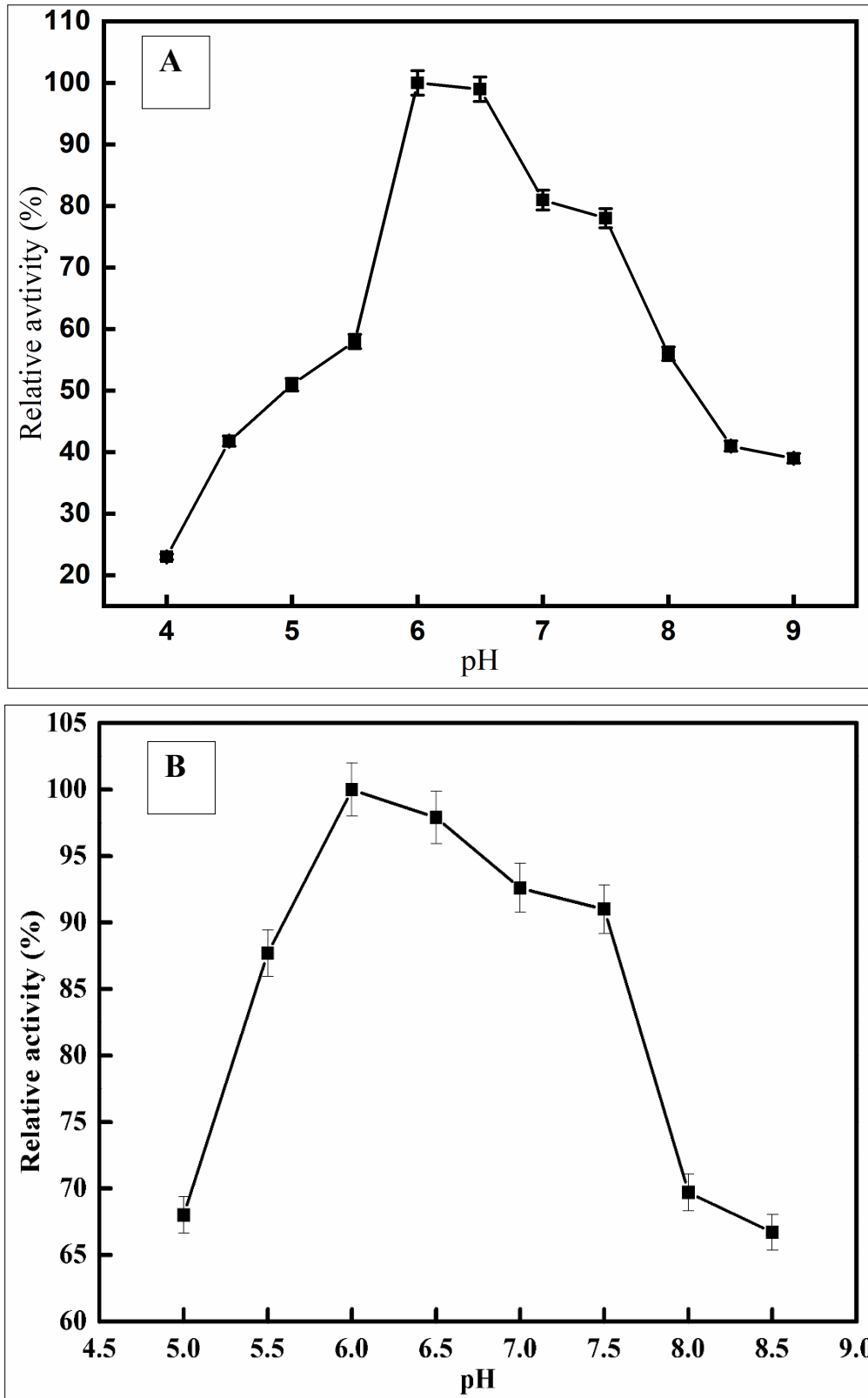


Figure 3. Optimum pH (A), pH stability (B)

Table 1. Purification scheme of *C. forskohlii* peroxidase

steps	T. units	T. Protein mg	S.A Unit/mg protein	Fold purification	Recovery 100%
Crude extract	725	11	66	1	100
Chromatography CM-sepharose					
0.0 M NaCl (POD1)	6.12	0.06	102	1.5	0.8
0.1M NaCl (POD 2)	9.18	0.15	61.2	0.9	1.2
0.2M NaCl (POD 3)	174	0.75	232	3.5	24
0.3M NaCl (POD 4)	20	0.10	200	3	2.8
Gel filtration on sephacryl S-200					
POD 3A	141	0.19	742	11	19

7.5 was relatively higher, which is compatible with the optimal pH study. The findings are in line with an earlier report where it was found that *Triticum aestivum* peroxidase was stable for two days at different pH (5.0-7.0) [22].

The temperature profile revealed the maximum *C. forskohlii* peroxidase activity at 50 °C temperature and steadily dropped above 50°C (Fig. 4a). The purified enzyme retained 63% and 40% of its initial activity at 60 and 70 °C, respectively. Similar findings have been reported for Arabian balsam peroxidase [8], *Spinacia oleracea* peroxidase [23]. The thermostability of the purified enzyme at various temperatures was shown in Fig. 4b. The enzyme was robust to 50 °C. At temperatures above 50 °C, the stability of the enzyme gradually decreased. A broad diversity in thermal stability for peroxidase activity has been noted from diverse sources, including date palm leaves (75 °C), [24] horseradish cv. Balady (40°C), [25], chewing stick miswak (40 °C) [26], and Arabian balsam (55 °C) [8].

The kinetic constants for guaiacol and Hydrogen peroxide oxidation by *C. Forskohlii* peroxidase has been calculated and the apparent K_m , K_{cat} and V_{max} values are shown in Table 2 (Fig.5 a,b). The *C. forskohlii* peroxidase provided K_m values of 3,355 and 5,395 mM for substrates H₂O₂ (Fig. 5a) and Guaiacol (Fig. 5b).

The K_m values appear to be less than those reported for *Triticum aestivum* peroxidase (7.3 and 6.6 mM for H₂O₂ and guaiacol) [27], peroxidase from Arabian balsam (4.81 and 46.5 mM for H₂O₂ and guaiacol) [8]. The low K_m values show a high evident affinity to H₂O₂ and guaiacol in the enzyme relative to previously reported peroxidases. The V_{max} values of *C. forskohlii* peroxidase was 1.531 and 1.242 $\mu\text{mole/ml}$ for H₂O₂ and guaiacol, respectively. On the other hand, the purified enzyme has K_{cat} values of 99.52 and 79.56 s⁻¹ for H₂O₂ and guaiacol, respectively. The catalytic efficiency (k_{cat}/K_m) of the purified enzyme using guaiacol and H₂O₂ was found to be 14.75 and 29.66 s⁻¹ mM⁻¹, respectively (Table 2).

The effects of metal ions (Cu²⁺, Co²⁺ and Ni²⁺), at a final concentration ranging from 2 to 10 mM, on *C. forskohlii* peroxidase activity are summarized in Table 3. Such metals have an activation effect on the *C. Forskohlia* peroxidase activity. Cu²⁺ and Ni²⁺ were reported to stimulate peroxidase activity in radish seedlings and Horseradish, respectively [28, 29].

The inhibitory effects of sodium azide, three metal ions and organic compounds of EDTA (2-10 mM), and urea (0.2-1 M) on *C. forskohlii* peroxidase activity are outlined in Table 4. When Sn²⁺ affectively inhibited purified peroxidase activity, metal ions Al³⁺ and Hg₂⁺

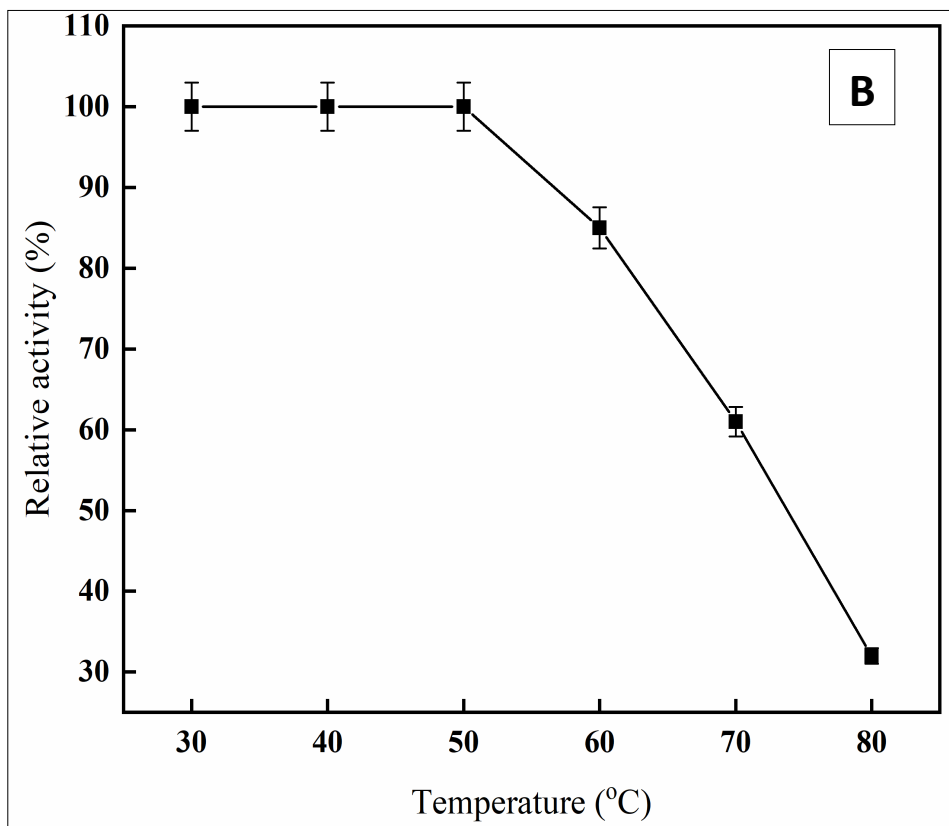
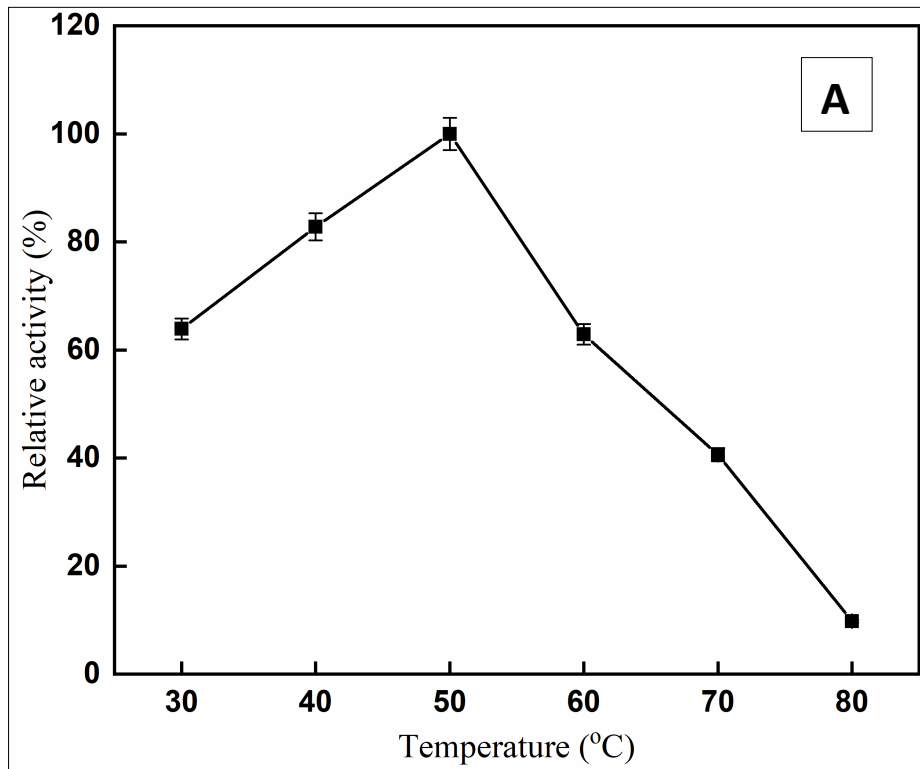


Figure 4. effect of temperature (A), Temperature Stability (B)

Table 2. Kinetic parameters of peroxidase from *Coleus forskohlii*

Kinetic parameter	<i>C. forskohlii</i> peroxidase	
	H ₂ O ₂	Guaiacol
Km (mM)	3.355	5.395
Vmax (μmole ml ⁻¹ min ⁻¹)	1.531	1.242
kcat (s ⁻¹)	99.52	79.56
kcat/Km (s ⁻¹ mM ⁻¹)	29.66	14.75

Table 3. Effect of metal salts on *C. forskohlii* peroxidase

Metal Salts	Relative activity %				
	2 mM	4 mM	6 mM	8 mM	10 mM
CuSO ₄	102.8	106.5	114.5	123.9	118.9
CoSO ₄	104.9	109.8	111.9	111.8	112.1
NiCl ₂	102.5	103.7	109.2	124.6	129.2

Table 4. Inhibition properties of some inhibitors on *C. forskohlii* peroxidase activity.

	Concentration	Ki (mM)		IC ₅₀ (mM)
		Guaiacol	H ₂ O ₂	
AlCl ₃	2-10 mM	0.41	1.01	3.83
SnCl ₂		0.072	0.179	1.38
Hg(NO ₃) ₂		0.79	0.196	5.49
NaN ₃		1.543	3.798	10.49
EDTA		1.187	2.952	9.15
Urea	0.2-1 M	0.094	0.233	0.808

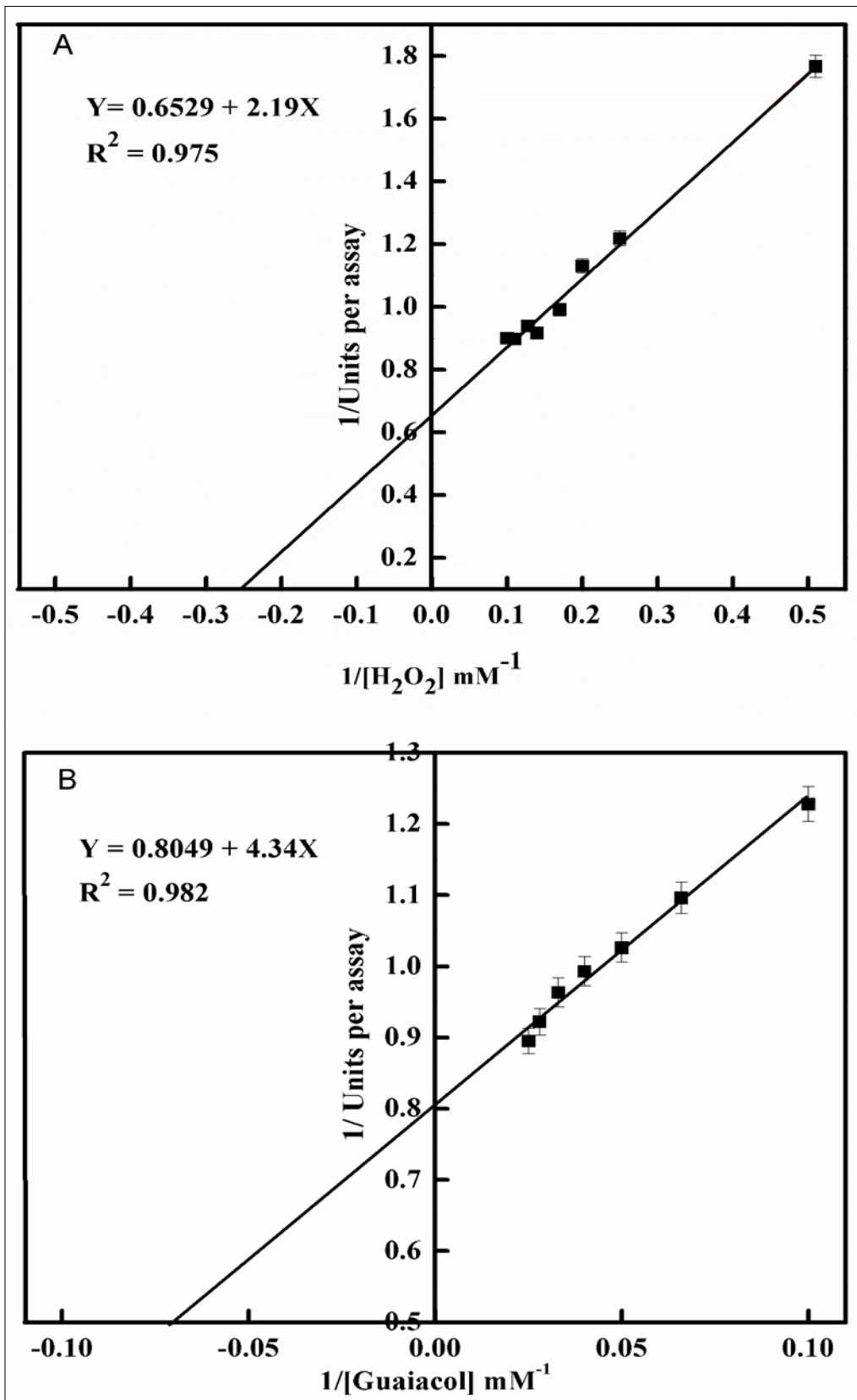


Figure 5. Lineweaver-Burk plots relating purified peroxidase reaction velocity to H₂O₂ (A) and guaiacol (B) concentrations.

showed moderate inhibition effects against peroxidase. Each of these metal ions had half maximum inhibition (IC₅₀) concentrations of 1.38, 3.83, and 5.49 mM, respectively. Furthermore, the inhibitor constant (K_i Guaiacol) of the specified metal was determined in accordance with the graph of Lineweaver–Burk, by 0.072, 0.41 and 0.79 mM and (K_i H₂O₂) by 0.179, 1.01 or 0.196 mM, respectively. Sodium azide and EDTA have moderate inhibitory effect on the purified peroxidase. The half maximum inhibition concentrations (IC₅₀) were calculated as 10.49 and 9.15 mM, respectively. while the inhibitor constant (K_i) were found to be 1.543, 1.187 mM for guaiacol and 3.798, 2.952 mM for H₂O₂, respectively. Inhibition of peroxidase by Sn²⁺, Al³⁺, Hg²⁺ ions, NaN₃, and EDTA were also reported in several studies [27, 30-32]. The direct interaction between a peptide backbone and urea molecule caused protein unfolding. Basically, high urea concentrations caused protein unfolding followed by decreased enzyme activity [33]. As shown in Table 4, exposure of purified peroxidase with urea at 0.2-1 M was determined. The half inhibition concentration (IC₅₀) was 0.808 mM, while the inhibitor constant (K_i) were 0.094 mM for Guaiacol and 0.233 mM for H₂O₂.

Conclusion

In this study, peroxidase was purified from a new source, *C. forskohlii*, using ion exchange chromatography (CM-Sephrose), and gel filtration chromatography (Sephacryl S-200). Appropriate conditions for the peroxidase activity have been analyzed and calculated. Peroxidase has been shown to be relatively active under acidic conditions. K_m, K_{cat}, K_i, V_{max} and IC₅₀ have been estimated for both substrates. The influences of different metal ions on peroxidase have also been determined. The enzyme performed optimal activity and stability in a temperature at 50 °C. The *C. forskohlii* peroxidase can potentially be used for bread leavening and bakery, in addition, high-temperature enzyme stability would be useful in these applications.

References

- Gülçin İ, Oktay M, Kireççi E, Küfrevioğlu Öİ, (2003) Screening of Antioxidant and Antimicrobial Activities of Anise (*Pimpinella anisum* L.) Seed Extracts. *Food Chem.* 83: 371–382.
- Oktay M, Gülçin İ, Küfrevioğlu Öİ, (2003) Determination of in Vitro Antioxidant Activity of Fennel (*Foeniculum vulgare*) Seed Extracts. *Lebensm Wissen Technol.* 36: 263–271.
- Shukla PK, Misra A, Kumar M, Jaichand SK, Akhtar J, Srivastava S, Agrawal PK, Singh Rawat, AK, (2018) Simultaneous quantification of forskolin and iso-forskolin in *Coleus forskohlii* (Wild.) Briq. and identification of elite chemotype, collected from eastern ghats (India). *Pharmacogn. Mag.* 13: S881–S885.
- Almulaiky YQ, Kuerban A, Aqlan F, Alzahrani SA., Baeshen MN, Afifi M, Alkhaled M, (2017) In vitro Antiglycation, Antioxidant Properties of *Coleus forskohlii*"Balady"Leaves and Stem and their Antioxidant Enzyme Activities. *Annu Res Rev Biol.* 1-11.
- Ding X, Staudinger JL, (2005) Induction of drug metabolism by forskolin, the role of the pregnane X receptor and the protein kinase a signal transduction pathway. *J. Pharmacol. Exp. Ther.* 312: 849–856.
- Zhang X, Li F, Guo L, Hei H, Tian L, Peng W, Cai H, (2015) Forskolin regulates Ltype calcium channel through interaction between actinin 4 and β3 subunit in osteoblasts. *PLoS One.* 10, e0124274.
- Köksal Z, Kalın R, GulçIn İ, Özdemir H, (2018) Inhibitory Effects of Selected Pesticides on Peroxidases Purified by Affinity Chromatography. *Int. J. Food Properties.* 21(1): 385–394.
- Almulaiky YQ, and Al-Harbi SA, (2019) A novel peroxidase from Arabian balsam (*Commiphora gileadensis*) stems: Its purification, characterization and immobilization on a carboxymethylcellulose/ Fe₃O₄ magnetic hybrid material. *Int. J. Biol. Macromol.* 133: 767-774.
- Cao XM, Zhang Y, Wang YT, Liao XJ, (2011) Effects of High Hydrostatic Pressure on Enzymes, Phenolic Compounds, Anthocyanins, Polymeric Color and Color of Strawberry Pulps. *J. Sci. Food Agric.* 91: 877–885.
- Chisari M, Barbagallo RN, Spagna G, (2007) Characterization of Polyphenol Oxidase and Peroxidase and Influence on Browning of Cold

- Stored Strawberry Fruit. *J. Agric. Food Chem.* 55: 3469–3476.
11. Gülçin İ, Yıldırım A, (2005) Purification and Characterization of Peroxidase from Brassica oleracea Var. Acephala. *Asian J. Chem.* 17(4): 2175–2183.
 12. Kharatmol PP, Pandit AB, (2012) Extraction, partial purification and characterization of acidic peroxidase from cabbage leaves. *J. Biochem Technol.* 4: 531–540.
 13. Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H, (2001) A large family of Class III plant peroxidases. *Plant Cell Physiology*, 42: 462–468.
 14. Hamid M, Rehman K, (2009) Potential applications of peroxidases. *Food Chem.* 115: 1177–1186.
 15. Bradford MM, (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72: 248–254.
 16. Laemmli UK, (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature.* 227: 680–685. DOI: 10.1038/227680a0.
 17. Yuan ZY, Jiang TJ, (2003) Horseradish peroxidase, in: JR Whitaker, A Voragen, DWS Wong (Eds.), *Handbook of food enzymology*, Marcel Dekker Inc., New York pp. 403–411.
 18. Kumar S, Satyanarayana T, (2003) Purification and kinetics of a raw starch hydrolyzing, thermostable, and neutral glucoamylase of the thermophilic mold *Thermomucor indicae-seudaticae*, *Biotechnol. Prog.* 19: 936–944.
 19. Goyal P, Chugh LK, (2013) Partial purification and characterization of peroxidase from pearl millet (*Pennisetum glaucum* [L.] r. br.) grains. *Journal of Food Biochem.* 38(2): 150–158.
 20. Deepa SS, Arumughan C, (2002). Purification and characterization of soluble peroxidase from oil palm (*Elaeis guineensis* jacq) leaf. *Phytochemistry*, 61(5): 503–511.
 21. Morisco F, Vitaglione P, Amoruso D, Russo B, Fogliano V, Caporaso N, (2008) Foods and liver health, *Mol. Asp. Med.* 29: 144–150.
 22. Altın S, Tohma H, Gülçin İ, Köksal E, (2017) Purification, characterization, and inhibition sensitivity of peroxidase from wheat (*Triticum aestivum* ssp. vulgare). *Int J Food Prop.* 20(9): 1949-1959.
 23. Köksal E, (2011). Peroxidase from leaves of spinach (*Spinacia oleracea*): Partial purification and some biochemical properties. *Int J Pharmaco.* 7(1): 135-139.
 24. Al-Senaïdy AM, Ismael MA, (2011) Purification and characterization of membrane-bound peroxidase from date palm leaves (*Phoenix dactylifera* L.). *Saudi J. Biol. Sci.*, 18(3), 293-298.
 25. Mohamed SA, Abulnaja KO, Ads AS, Khan JA, Kumosani TA, (2011). Characterisation of an anionic peroxidase from horseradish cv. Balady. *Food Chem.* 128(3), 725-730.
 26. Mohamed SA, Al-Malki AL, Khan JA, Sulaiman MI, Kumosani TA, (2012) Properties of peroxidase from chewing stick miswak. *Afr J Pharm Pharmacol.* 6(9): 660-670.
 27. Altay A, Koktepe T, Durmaz L, Topal F, Gülçin İ, Köksal E, (2018) Purification and selected biochemical properties of peroxidase from cress (*Lepidium sativum* sub sp. sativum). *Int. J. Food Prop.* 21(1): 2610-2621.
 28. Chen EL, Chen YA, Chen LM, Liu ZH, (2002) Effect of copper on peroxidase activity and lignin content in *Raphanus sativus*. *Plant Physiology and Biochemistry: PPB / Societe Francaise De Physiologie Vegetale*, 40: 439–444.
 29. Nazari K, Mahmoudi A, Khodafarin R, Moosavi-Movahedi AA, Mohebi A, (2005) Stabilizing and suicide-peroxide protecting effect of Ni²⁺ on horseradish peroxidase. *J Iran Chem Soc.*2(3), 232-237.
 30. Yuzugullu Karakus Y, Acemi A, Işık S, Duman Y, (2018) Purification of peroxidase from *Amsonia orientalis* by three-phase partitioning and its biochemical characterization. *Sep Sci Technol.* 53 (5): 756-766.
 31. Alshawafi WM, Aldahri M, Almulaiky YQ, Salah N, Moselhy SS, Ibrahim IH, Mohamed SA. (2018) Immobilization of horseradish peroxidase on PMMA

- nanofibers incorporated with nanodiamond. *Artif Cells Nanomed Biotechnol* 46(sup3), S973-S981.
32. Bilal M, Asgher M, Ramzan M, (2015) Purification and biochemical characterization of extracellular manganese peroxidase from *Ganoderma lucidum* IBL-05 and its application. *Scientific Research and Enzyme Essays*. 10(14): 456-464.
33. Akhtar S, Khan AA, Husain QJ. Simultaneous purification and immobilization of bitter melon (*Momordica charantia*) peroxidases on bioaffinity support. *J Chem Technol Biotechnol*. 2005; 80: 198–205.