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Research Article

One-step isolation and purification of peroxidase from zucchini heads

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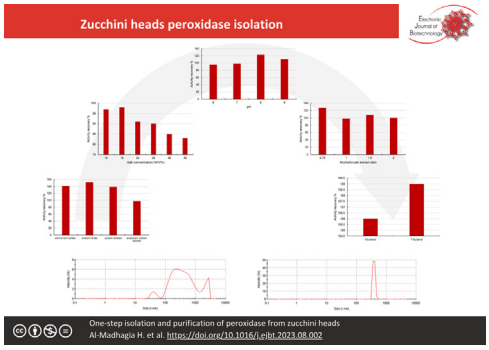
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Food and agricultural wastes are produced in huge amounts yearly, putting extra effort into their removal or valorization. One of these wastes is the zucchini heads (ZH) which, in this study, was used as a source to isolate peroxidase using three-phase portioning (TPP).
Results: Different parameters of TPP were optimized to ensure obtaining the maximal activity recovery and purity of the enzyme. The purity of the isolated enzyme was performed using the protein homogeneity module of dynamic light scattering. This was followed by the determination of the optimal pH and temperature of the isolated ZH peroxidase. It was found that sodium citrate at a concentration of 15%, pH 8, 1-butanol as the upper alcoholic phase, and an alcohol/crude extract ratio of 0.75:1 were the best conditions for ZH peroxidase isolation. The obtained activity recovery and purification fold were 159% and 10.05, respectively. The isolated ZH peroxidase displayed a high purity as emphasized via dynamic light scattering. The optimum pH and temperature were 8 and 25°C.
Conclusions: The present study was the first to isolate and purify peroxidase from ZH using TPP in one step.
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1. Introduction

Peroxidase enzymes are a family of oxidoreductase enzymes that are capable of catalyzing the transfer of electrons from hydrogen peroxide or organic hydroperoxides to a variety of both organic

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and inorganic substrates [1]. They exhibit wide distribution in living organisms, including plants, animals, and microorganisms, and play important roles in many physiological processes such as defense against pathogens, lignin biosynthesis, and hormone metabolism. Plant peroxidase and polyphenol oxidase are responsible for the browning reaction taking place in different fruits after cutting some parts of them [2]. Structurally, peroxidases are heme-containing enzymes that contain a prosthetic group composed of a central iron atom coordinated to a protoporphyrin IX molecule. The heme group provides peroxidase with the redox ability to catalyze the oxidation of various substrates [3]. Peroxidases can be classified into several classes based on their structural features, including plant peroxidases, animal peroxidases, and fungal peroxidases. They can also be classified based on their mechanisms of action, such as classical peroxidases, catalases, and peroxiredoxins. In plants, peroxidases play a critical role in lignification, a process that strengthens cell walls and provides structural support for the plant. In animals, peroxidases are involved in the immune response and can help protect against oxidative stress [4,5]. Concerning its active site, it has a large pocket that accepts a wide range of substrates rendering it a versatile enzyme [6]. Given the fact that peroxidase can accept various substrates, it has a range of applications in biotechnology, including the bioremediation of environmental micropollutants and the development of analytical biosensors [7]. Peroxidases can be used to break down pollutants in wastewater, soil, and air, making them a superior tool for environmental green cleaning [8]. Additionally, peroxidases can be used in the development of biosensors for the detection of various analytes, including glucose [9], cholesterol [10], and hydrogen peroxide [11].

Indeed, commercial peroxidase can be purchased from various industrial companies. This peroxidase is conventionally isolated from horseradish using ion-exchange chromatography [12,13]. However, its high cost urged scientists to search for cheaper sources, particularly food wastes [14]. For example, Almulaiky [15] isolated and purified peroxidase enzyme from *Coleus forskohlii* using ion-exchange and gel filtration techniques with 11-fold purification. Similarly, *Ziziphus jujuba* fruit was another source for peroxidase with 18.9-fold enhancement of activity with a recovery of 20% [16]. In a similar fashion, peroxidase was also extracted and purified from Arabian balsam using the same techniques and immobilized onto carboxymethycellulose/Fe_3O_4 hybrid for enhanced activity and reusability for 15 catalytic cycles [17].

TPP (Three-phase partitioning) is a valuable bioseparation technique, offering efficiency, cost-effectiveness, and user-friendly operation. With its ability to yield high activity as well as purity of isolated biomolecules and facilitate the recovery of desired enzymes, TPP has proven to be an indispensable tool in the field of bioseparation. Furthermore, many reports emphasized the replacement of TP with the traditional chromatographic techniques for isolating and purifying enzymes in particular [18,19]. This is the first study to isolate peroxidase from zucchini heads using TPP.

2. Materials and methods

The multi-process workflow of the current study was illustrated in Fig. 1. However, the details of each step are explained below.

2.1. Reagents

Sodium dihydrogen phosphate, disodium hydrogen phosphate, ammonium sulfate, and potassium sodium tartrate were purchased from Merck (99%) whereas sodium citrate, sodium acetate, and t-butanol were purchased from HiMedia (97%) and lactalbumin from Sigma (99%). Phosphate buffer used was prepared at pH 7 and 100 mM concentration.

2.2. Plant preparation and processing

Fresh zucchini was purchased from the local market of Aleppo City, Syria. The zucchini heads (ZH) were excised from the rest of the plant and washed twice with distilled water. Then, 10 g of the ZH together with 100 ml of cold phosphate buffer (pH 7) was homogenized using a commercial blender. It should be noted here that the buffer used was cold and the blending process has been conducted in intermittently to avoid enzyme denaturation. Afterward, filtration and subsequent centrifugation at 5000 rpm for 20 min were done and the supernatant was stored at 4°C to be used later.

2.3. Preparation of TPP

In a 15 ml test tube, 5 ml of the crude extract was mixed with different salt concentrations. Then, various volumes of alcohol were added to the tube and vigorously mixed and settled for 3 h. After that, a brief centrifugation step was conducted to ease the separation of the three layers (3000 rpm for 5 min). The enzymatic activity was measured for the bottom layer (aqueous phase). However, many parameters were optimized to achieve maximum separation efficiency involving different salt types, salt concentrations, pH degrees, different alcohol types, and varying the alcohol/crude extract ratios [20].

2.4. Determination of peroxidase assay

We followed the method described by Yuzugullu Karakus et al. [21]. In brief, approximately 2.4 ml of phosphate buffer (pH 7) was added to a cuvette, followed by the addition of 300 µl of 5.3% pyrogallol, and 200 µl of 0.6% hydrogen peroxide. The reaction was initiated with the addition of 100 µl of the corresponding enzyme source (total volume of 3 ml) at 420 nm for 4 min which was monitored.

2.5. Determination of protein content

The concentration of protein of the crude extract and the isolated enzyme was performed according to the modified Lowry method [22]. A concentration range of 0.1–0.4 mg/ml of lactalbumin was utilized for plotting the standard curve.

2.6. Assessment of purity

To check the efficiency of the isolated ZH peroxidase, the protein homogeneity module of dynamic light scattering (DLS) was used. DLS protein homogeneity is a well-known method for evaluating the purity as well as homogeneity of the isolated proteins, expressed recombinant protein, and the produced monoclonal antibody [23]. The purity of the isolated enzyme was compared to the crude extract.

2.7. Determination of optimum pH and temperature

Various temperatures (15–35°C) were tested for the potential influence on the isolated ZH peroxidase. Likewise, to determine the optimal pH of the isolated enzyme, a pH range of 5–9 was examined. The temperature and pH effects were analyzed by monitoring change in enzymic activity.

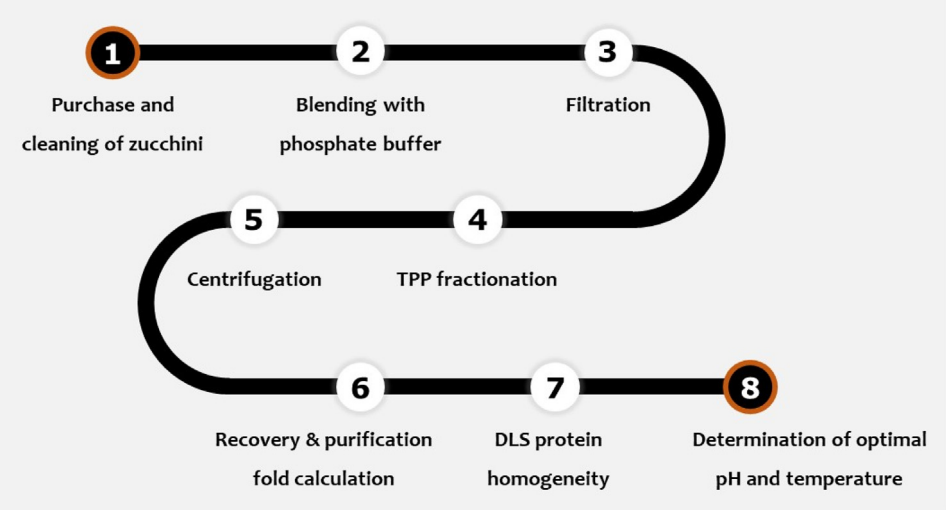

Fig. 1. The designed workflow of this multi-step study.

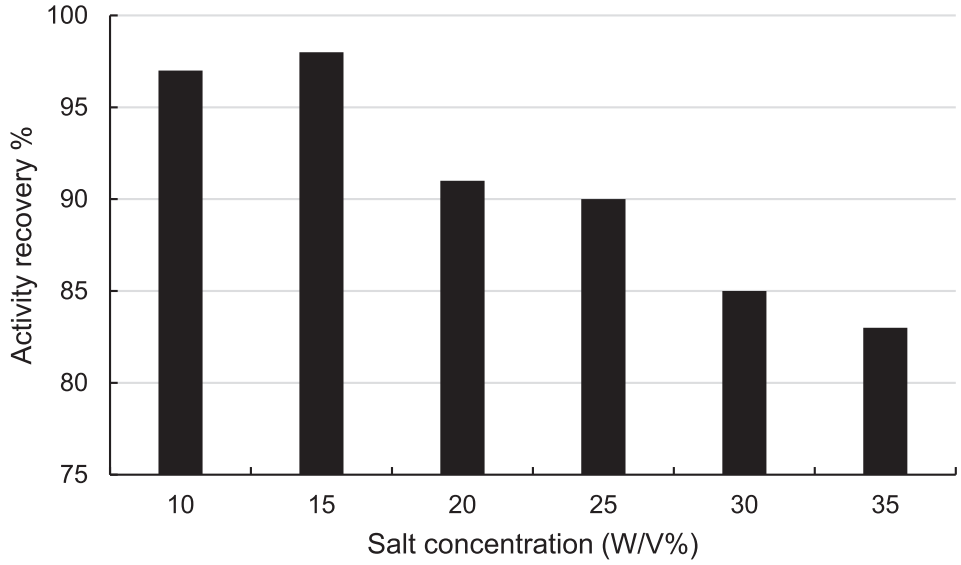

Fig. 2. Effect of salt concentration on the ZH peroxidase separation efficiency. The salt type was potassium sodium tartrate, pH of 7 and alcohol/crude extract ratio of 1.

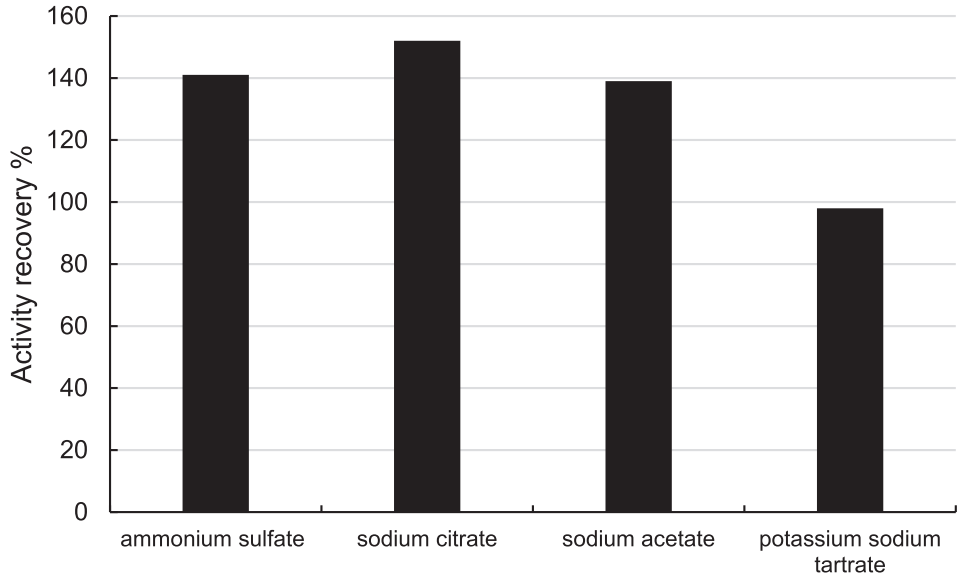

Fig. 3. Effect of salt type on the ZH peroxidase activity recovery. The salt concentration was set at 15% w/v, pH at 7, and alcohol/crude extract ratio at 1.

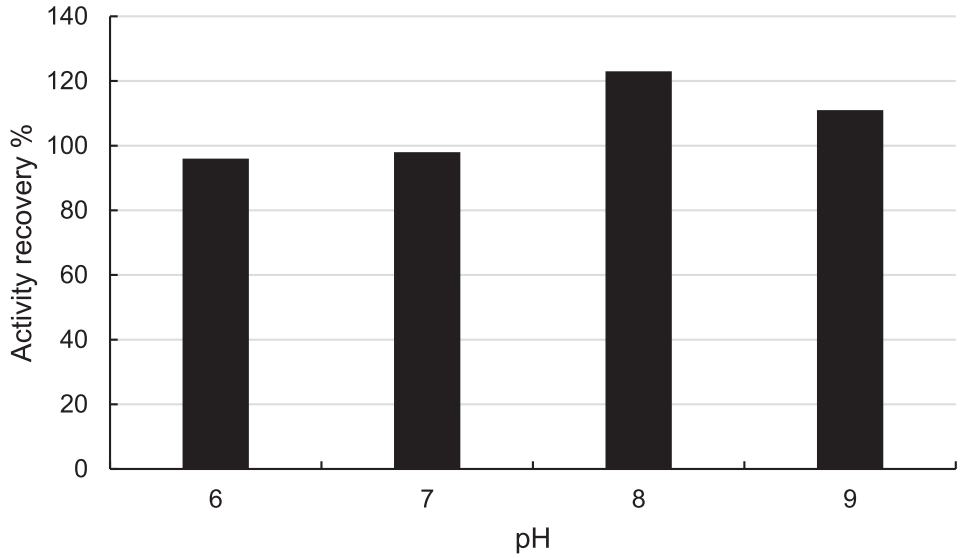

Fig. 4. Effect of pH on ZH peroxidase activity recovery. The salt type was sodium citrate (15% w/v) and the alcohol/crude extract ratio at 1.

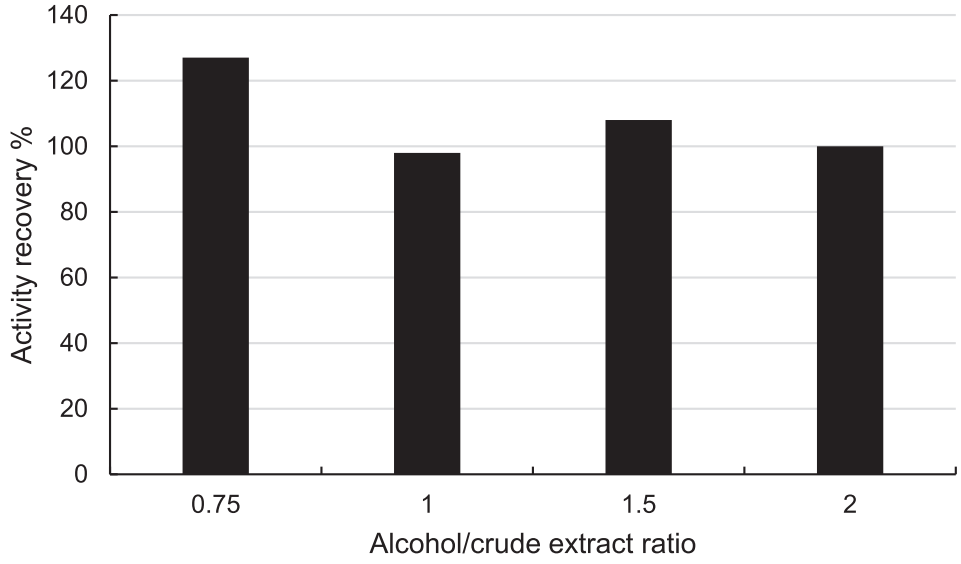

Fig. 5. Effect of alcohol/crude extract ratio on ZH peroxidase activity recovery. The salt type was sodium citrate (15% w/v), and the pH at 7.

2.8. Data analysis

The activity recovery and purification fold were calculated according to the following formulas:

$$Activity\ recovery\ \% = \frac{activity\ of\ isolated\ peroxidase}{activity\ of\ crude\ extract} \times 100$$

$$Purity\ fold = \frac{Specific\ activity\ of\ isolated\ peroxidase}{Specific\ activity\ of\ crude\ extract}$$

The results of the current study were processed and analyzed via Microsoft Excel® 2019. The same program was utilized for the graphical representation of results as well as the regression analysis.

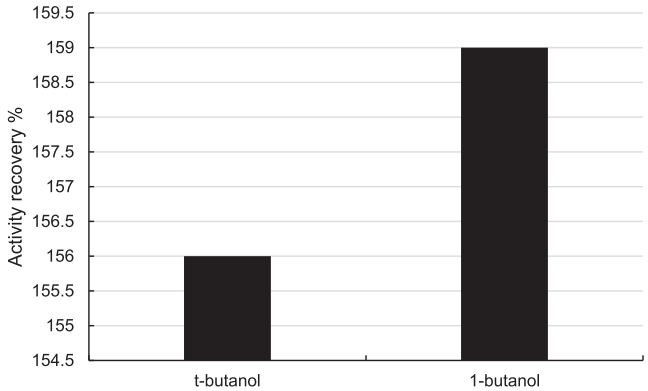

Fig. 6. Effect of alcohol type on ZH peroxidase separation efficiency. The salt type was sodium citrate (15% w/v), with pH of 8 and alcohol/crude extract ratio of 0.75.

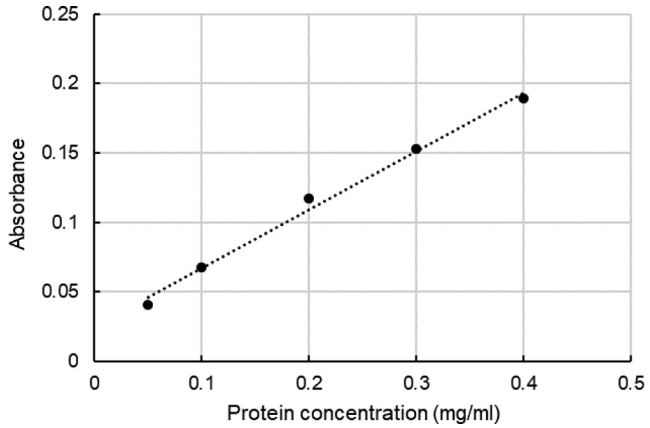

Fig. 7. Standard curve established for calculation of protein content.

3. Results

3.1. Effect of salt concentration

The first step was to screen the best concentration of salt that gives maximum enzymatic recovery via salting-out. It turned out that at a concentration of 15% w/v sodium citrate, ZH peroxidase recovery was maximum (98%). However, after this concentration, the recovery declines gradually (Fig. 2).

3.2. Effect of salt type

As shown in Fig. 3, sodium citrate was the best salt fractionating ZH peroxidase with an activity recovery of 152%, followed by ammonium sulfate and sodium acetate (141% and 139%).

3.3. Effect of pH

A pH range from 6 to 9 was examined. As the pH increases, activity recovery increases till to reach maximum at pH 8 (123%). Afterward, the pH decreases to 111% as depicted in Fig. 4.

3.4. Effect of alcohol/crude extract ratio

The next step was to evaluate the influence of changing the ratio between the upper layer (alcohol) and the lower layer (aqueous). It was found that at the ratio of 0.75, the highest activity recovery was attained (127%). Raising the ratio above this value posed a negative impact on the fractionation yield as shown in Fig. 5.

3.5. Effect of alcohol type

Two types of alcohols were tested, i.e. t-butanol (standard alcohol in TPP) and 1-butanol. Unexpectedly, 1-butanol had a slightly higher positive impact on the activity recovery of ZH peroxidase than the standard alcohol t-butanol (159% vs 156%) as depicted in Fig. 6.

3.6. Determination of protein content

After detecting the optimal conditions for isolating ZH peroxidase using TPP, all these conditions were combined to get the highest yield of enzyme isolation efficiency. A standard curve from lactalbumin was established to infer the corresponding protein concentration (Fig. 7). The standard curve had a coefficient of correlation (R^2) of 0.9921 denoting the near-optimal correlation. The protein content of the combined optimized yield with activity recovery was utilized for calculating specific activity and purification fold of ZH peroxidase as summarized in Table 1.

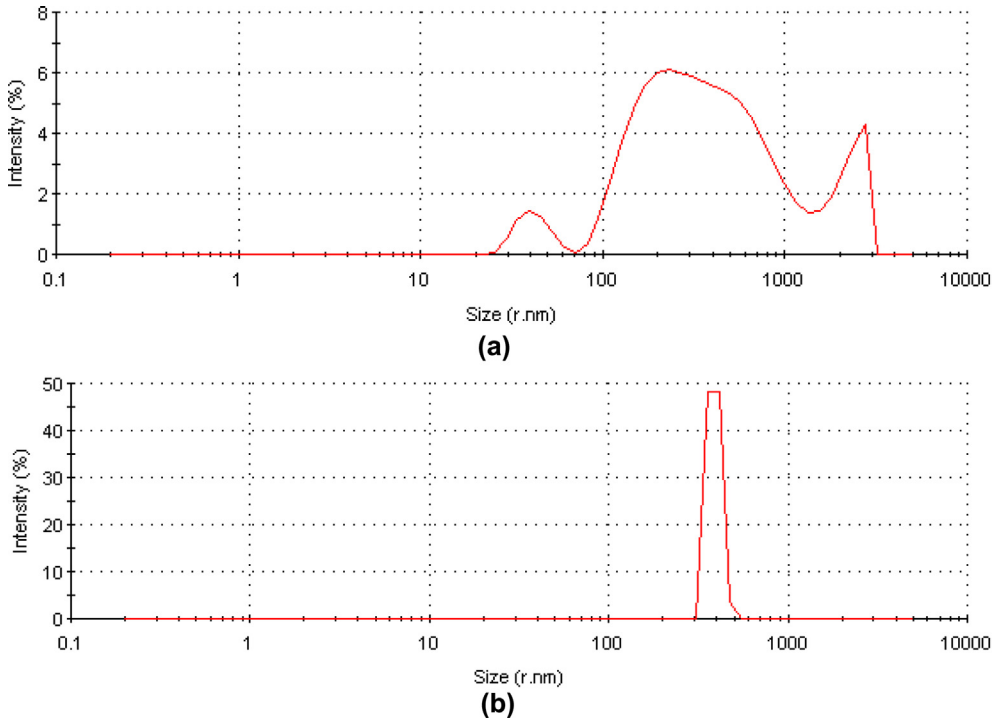

Fig. 8. DLS protein homogeneity of the crude ZH extract (a) and the isolated enzyme (b).

Table 1
Specific activity, activity recovery, and purification fold of the isolated ZH peroxidase.

Enzyme	Activity recovery	Specific activity	Purification fold
ZH peroxidase	159% ± 3	5600 U/mg ± 64	10.05 ± 0.8

Results were performed in duplicate and expressed as mean ± standard deviation.

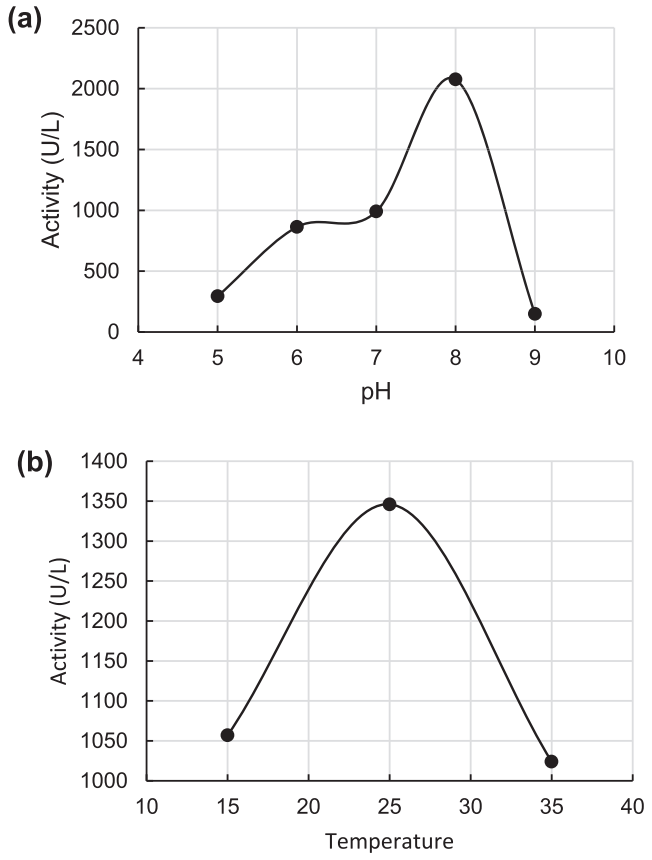

Fig. 9. Optimum pH (a) and temperature (b) of the isolated ZH peroxidase.

3.7. Assessment of purity

It is well-known that as the purification fold increases, the likelihood of increasing the actual purity of the enzyme increases accordingly. This is what has been obtained during the assessment of the purity of the isolated ZH peroxidase using DLS (Fig. 8). The crude extract displayed three peaks with different diameters, indicative of the heterogeneity of the sample composition. On the flip side, the isolated ZH peroxidase exhibited only a single peak with high intensity. Moreover, this single peak had a very narrow polydispersity index indicating the high purity of the isolated enzyme.

3.8. Optimal pH and temperature

The optimal pH and optimal temperature of the isolated ZH peroxidase were analyzed (Fig. 9). The pH of ZH peroxidase gradually increases until it reaches a threshold at pH 8 (2075 U/L) reflecting the slightly alkaline properties. Nonetheless, a substantial drop in activity was observed after this optimal value. Similarly, the influence of temperature on the activity of isolated ZH peroxidase exhibited a bell-like curve where a temperature of 25°C was the optimum temperature. This classified the isolated ZH peroxidase as a sensitive enzyme.

Table 2
Activity recovery and purification fold of some plant sources for peroxidase isolation using TPP.

Enzyme source	Activity recovery %	Purification folds	Reference
Amsonia orientalis	162%	12.5	[21]
Ipomoea palmata	81%	18	[20]
Citrus sinenses	93.96%	18.20	[33]
Orange peels	91.84%	24.28	[34]
Momordica charantia	170%	4.84	[35]
Zucchini heads	159%	10.05	Current study

4. Discussion

Food and agricultural wastes involve a large group of residual materials generated during/after agricultural activities such as coffee pulp from the coffee industry, husks from the cereal industry, and peels from the starch-based industry [24]. Indeed, huge amounts of these wastes are generated year after year requiring downstream processing for simultaneous removal of the concomitating adverse effects and valorization of such wastes. Agro-industrial wastes have been valorized to be a rich resource for enzymes, biofuels, vitamins, antioxidants, antimicrobials, and animal feed [25].

TPP is a powerful bioseparation technique that offers numerous benefits for isolating biomolecules, particularly enzymes, from crude extracts. It has consistently proved its efficacy in terms of achieving high yields of isolated biomolecules. One of the key advantages of TPP as a fractionation method is its ability to operate under mild conditions, making it highly efficient and cost-effective. Moreover, TPP has the potential for the recycling of chemicals used, minimizing waste, and thus can be considered a green tool. Additionally, TPP is a time-saving technique that offers a rapid approach to bioseparation which precedes the routinely used chromatographic counterparts [26]. TPP is fractionating components within crude extracts into three phases, i.e. lipids and nonpolar substances can be effectively separated and moved to the t-butanol layer, while certain proteins aggregate at the interface layer retaining the lower layer (aqueous phase) rich in the polar biomolecules. This unique characteristic of TPP contributes significantly to the recovery of the desired enzyme, enhancing the overall efficiency of the separation process [27]. Notably, TPP has successfully been utilized to isolate a wide range of enzymes from diverse sources and waste materials. Prominent examples include the isolation of lipase [28], bromelain [29], papain [30], and other proteases [31], all of which have demonstrated improved activity and purity through the application of TPP.

TPP parameters had to be optimized to get the highest possible enzyme recovery and purification fold. This involves precipitating salt type, salt concentration, pH, and ratio between alcohol and crude extract in addition to the type of alcohol used. When all optimized conditions were combined, an activity recovery and

purification fold of 159% and 10.05 of ZH peroxidase were obtained in the bottom phase whereas other proteins and phenolic compounds were accumulated in the middle and upper phases, respectively. This denotes that almost all of the enzyme has been moved to the aqueous phase reflecting the high separation efficiency. In addition, the best-extracting salt (sodium citrate) had a slight activating action of peroxidase activity which account for the extra in the percentage over 100%. An optimized alcohol/crude extract ratio of 0.75 indicates the low nonpolar moieties in the extract needed to be fractioned to concentrate the enzyme in the bottom layer [32]. Several researchers reported the application of TPP to isolate and purify peroxidase from different sources. For example, Vetal and Rathod [33] upon applying the optimized TPP setup for peroxidase separation from orange peels, the obtained recovery percentage of the enzyme was 93% after 180 min. Yuzugullu Karakus et al. [21] purified peroxidase enzyme from the flowering plant *Amsonia orientalis* via the TPP tool. However, 162% activity recovery was found after only 30 min with more than 18-fold purity. Moreover, TPP can be coupled with an ultrasonicator instrument to further reducing the time of enzyme fractionation. This approach was employed to purify peroxidase enzyme from orange peels with activity recovery and purification fold of 91% and 24.28 within only 6 min [34]. Indeed, our findings exhibited acceptable agreement with these reports. Table 2 summarizes the isolation of peroxidase from different sources using TPP.

5. Conclusions

This was an unprecedented study utilizing the TPP tool to isolate peroxidase from ZH. The optimized conditions for the isolation were as follows: 15% sodium citrate for salting-out, pH 8, alcohol/crude extract ratio of 0.75, and 1-butanol as the upper alcoholic phase. This yielded an activity recovery of 159% along with a purification fold of 10.05. This suggested the potential high purity of the isolated enzyme. Moreover, an assessment of the purity was conducted by DLS protein homogeneity measurement which confirmed a highly purified enzyme. The optimal pH and temperature of the isolated ZH peroxidase were 8 and 25°C, respectively. Further consideration of the isolated ZH peroxidase for the possible applications should find its way.

Author contributions

- Study conception and design: H. Al-Madhagi; V. Yazbik
- Data collection: H. Al-Madhagi; W. Abdelwahed
- Analysis and interpretation of results: H. Al-Madhagi; V. Yazbik
- Draft manuscript preparation: H. Al-Madhagi; W. Abdelwahed
- Revision of the results and approval of the final version of the manuscript: V. Yazbik; W. Abdelwahed.

Conflict of interest

The authors declare no competing interests.

Data availability

Data are available upon request.

References

[1] de Oliveira FK, Santos LO, Buffon JG. Mechanism of action, sources, and application of peroxidases. Food Res Int 2021;143:110266. https://doi.org/10.1016/j.foodres.2021.110266. PMid: 33992367.
[2] Passardi F, Longet D, Penel C, et al. The class III peroxidase multigenic family in rice and its evolution in land plants. Phytochemistry 2004;65(13):1879–93. https://doi.org/10.1016/j.phytochem.2004.06.023. PMid: 15279994.
[3] Patil PD, Nadar SS, Marghade DT. Photo-enzymatic green synthesis: The potential of combining photo-catalysis and enzymes. In: Inamuddin, Boddula R, Ahamed MI, editors. Advances in green synthesis: Advances in Science, Technology & Innovation. Springer International Publishing; 2021. p. 173–89. https://doi.org/10.1007/978-3-030-67884-5_9.
[4] Martínez AT, Ruiz-Dueñas FJ, Martínez MJ, et al. Enzymatic delignification of plant cell wall: from nature to mill. Curr Opin Biotechnol 2009;20(3):348–57. https://doi.org/10.1016/j.copbio.2009.05.002. PMid: 19502047.
[5] Gan J, Bilal M, Li X, et al. Peroxidases-based enticing biotechnological platforms for biodegradation and biotransformation of emerging contaminants. Chemosphere 2022;307(Part 3):136035. https://doi.org/10.1016/j.chemosphere.2022.136035. PMid: 35973503.
[6] Bilal M, Zdarta J, Jesionowski T, et al. Manganese peroxidases as robust biocatalytic tool — An overview of sources, immobilization, and biotechnological applications. Int J Biol Macromol 2023;234:123531. https://doi.org/10.1016/j.ijbiomac.2023.123531. PMid: 36754266.
[7] Pandey VP, Awasthi M, Singh S, et al. A comprehensive review on function and application of plant peroxidases. Biochem Anal Biochem 2017;6:308. https://doi.org/10.4172/2161-1009.1000308.
[8] Basumatary D, Yadav HS, Yadav M. The role of peroxidases in the bioremediation of organic pollutants. Nat Prod J 2023;13(1):60–77. https://doi.org/10.2174/2210315512666220410132847.
[9] Amor-Gutiérrez O, Costa Rama E, Costa-García A, et al. Paper-based maskless enzymatic sensor for glucose determination combining ink and wire electrodes. Biosensors Bioelectron 2017;93:40–5. https://doi.org/10.1016/j.bios.2016.11.008. PMid: 27856164.
[10] Rastogi L, Dash K, Sashidhar RB. Selective and sensitive detection of cholesterol using intrinsic peroxidase-like activity of biogenic palladium nanoparticles. Curr Res Biotechnol 2021;3:42–8. https://doi.org/10.1016/j.crbiot.2021.02.001.
[11] Singh G, Kushwaha A, Sharma M. Intriguing peroxidase-mimic for H_2O_2 and glucose sensing: A synergistic $Ce_2(MoO_4)_3$/rGO nanocomposites. J Alloy Compd 2020;825:154134. https://doi.org/10.1016/j.jallcom.2020.154134.
[12] Veitch NC. Horseradish peroxidase: A modern view of a classic enzyme. Phytochemistry 2004;65(3):249–59. https://doi.org/10.1016/j.phytochem.2003.10.022. PMid: 14751298.
[13] Mohamed SA, Abulnaja KO, Ads AS, et al. Characterisation of an anionic peroxidase from horseradish cv. Balady Food Chem 2011;128(3):725–30. https://doi.org/10.1016/j.foodchem.2011.03.096.
[14] Krainer FW, Glieder A. An updated view on horseradish peroxidases: Recombinant production and biotechnological applications. Appl Microbiol Biotechnol 2015;99:1611–25. https://doi.org/10.1007/s00253-014-6346-7. PMid: 25575885.
[15] Almulaiky YQ. Peroxidase from *Coleus forskohlii*: purification and biochemical characterization. IJN 2020;5(1):9–20. https://doi.org/10.14302/issn.2379-7835.ijn-19-3139.
[16] Zeyadi M, Almulaiky YQ. A novel peroxidase from *Ziziphus jujuba* fruit: Purification, thermodynamics and biochemical characterization properties. Sci Rep 2020;10:8007. https://doi.org/10.1038/s41598-020-64599-9. PMid: 32409642.
[17] Almulaiky YQ, Al-Harbi SA. A novel peroxidase from Arabian balsam (*Commiphora gileadensis*) stems: Its purification, characterization and immobilization on a carboxymethylcellulose/Fe_3O_4 magnetic hybrid material. Int J Biol Macromol 2019;133:767–1174. https://doi.org/10.1016/j.ijbiomac.2019.04.119. PMid: 31004641.
[18] Yan J-K, Wang Y-Y, Qiu W-Y, et al. Three-phase partitioning as an elegant and versatile platform applied to nonchromatographic bioseparation processes. Crit Rev Food Sci Nutr 2018;58(14):2416–31. https://doi.org/10.1080/10408398.2017.1327418. PMid: 28609145.
[19] Chew KW, Ling TC, Show PL. Recent developments and applications of three-phase partitioning for the recovery of proteins. Sep Purif Rev 2019;48(1):52–64. https://doi.org/10.1080/15422119.2018.1427596.
[20] Narayan AV, Madhusudhan MC, Raghavarao KSMS. Extraction and purification of *Ipomoea* peroxidase employing three-phase partitioning. Appl Biochem Biotechnol 2008;151:263–72. https://doi.org/10.1007/s12010-008-8185-4. PMid: 18369532.
[21] Yuzugullu Karakus Y, Acemi A, Işık S, et al. Purification of peroxidase from *Amsonia orientalis* by three-phase partitioning and its biochemical characterization. Sep Sci Technol 2018;53(5):756–66. https://doi.org/10.1080/01496395.2017.1405990.
[22] Markwell MAK, Haas SM, Bieber LL, et al. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978;87(1):206–10. https://doi.org/10.1016/0003-2697(78)90586-9. PMid: 98070.
[23] Stetefeld J, McKenna SA, Patel TR. Dynamic light scattering: a practical guide and applications in biomedical sciences. Biophys Rev 2016;8:409–27. https://doi.org/10.1007/s12551-016-0218-6. PMid: 28510011.
[24] Sadh PK, Duhan S, Duhan JS. Agro-industrial wastes and their utilization using solid state fermentation: a review. BioResourc Bioprocess 2018;5:1. https://doi.org/10.1186/s40643-017-0187-z.
[25] Yusree FIFM, Peter AP, Mohd Nor MZ, et al. Latest advances in protein-recovery technologies from agricultural waste. Foods 2021;10(11):2748. https://doi.org/10.3390/foods10112748. PMid: 34829028.
[26] Vidhate GS, Singhal RS. Extraction of cocoa butter alternative from *kokum* (*Garcinia indica*) kernel by three phase partitioning. J Food Eng 2013;117(4):464–6. https://doi.org/10.1016/j.jfoodeng.2012.10.051.

[27] Ketnawa S, Rungraeng N, Rawdkuen S. Phase partitioning for enzyme separation: An overview and recent applications. Int Food Res J 2017;24 (1):1–24.
[28] Kublicki M, Koszelewski D, Brodzka A, et al. Wheat germ lipase: Isolation, purification and applications. Crit Rev Biotechnol 2022;42(2):184–200. https://doi.org/10.1080/07388551.2021.1939259. PMid: 34266327.
[29] Gul A, Khan S, Arain H, et al. Three-phase partitioning as an efficient one-step method for the extraction and purification of bromelain from pineapple crown waste. J Food Process Preserv 2022;46(11):e16973.
[30] Jain J. Review on isolation and purification of papain enzyme from papaya fruit. Int J Eng Appl Sci Technol 2020;5(6):193–7. https://doi.org/10.33564/IJEAST.2020.v05i06.028.
[31] Eyssen LE, Goldring JPD, Coetzer THT. Three-phase partitioning (TPP) of proteases from parasites, plants, tissue and bacteria for enhanced activity. In: Gupta M, Roy I, editors. Three Phase Partitioning. Elsevier; 2021. p. 133–54. https://doi.org/10.1016/B978-0-12-824418-0.00008-4.
[32] Gagaoua M, Hafid K. Three phase partitioning system, an emerging non-chromatographic tool for proteolytic enzymes recovery and purification. Biosensors J 2016;5(1):100134.
[33] Vetal MD, Rathod VK. Three phase partitioning a novel technique for purification of peroxidase from orange peels (*Citrus sinenses*). Food Bioprod Process 2015;94:284–9. https://doi.org/10.1016/j.fbp.2014.03.007.
[34] Vetal MD, Rathod VK. Ultrasound assisted three phase partitioning of peroxidase from waste orange peels. Green Process Synth, 2016;5 (2):205–12. https://doi.org/10.1515/gps-2015-0116.
[35] Panadare DC, Rathod VK. Extraction of peroxidase from bitter gourd (*Momordica charantia*) by three phase partitioning with dimethyl carbonate (DMC) as organic phase. Process Biochem 2017;61:195–201. https://doi.org/10.1016/j.procbio.2017.06.028.