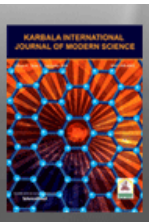

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An economical source for peroxidase: maize cobs

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An economical source for peroxidase: maize cobs

Abstract

According to the current situation of peroxidase (POD), the relevant studies on this enzyme indicated its importance as a tool in clinical biochemistry and different industrial fields. Most of these studies used the fruits and vegetables as source of this enzyme. So that in order to couple the growing requirements for POD with the recent demands for reduc-ing disposal volume by recycling the plant waste, the aim of the present study was to extract POD through management of municipal bio-waste of Iraqi maize species. A simple, green and economical method was used to extract this enzyme. Our results revealed that maize cobs are rich sources of POD, where the activity of this enzyme was found to be 7035.54 U/g of cobs. In pilot experiments this enzyme was extracted from the cobs using an efficient extraction buffer with either Cetyl Trimethyl Ammonium Bromide (CTAB), or sonication. To purify the extracted enzyme the previous step was followed by aqueous two phase extraction (ATPE) using 20% (w/v) polyethylene glycol (PEG) and 9% (w/v) ammonium sulfate. The obtained results indicated that POD was partially purified with 2.36 fold of purification and 81.78% recovery. The optimum temperature and pH of the extracted POD activity as well as the enzyme thermal stabil-ity were determined and found to be 20°C, pH 6, and stable at 60°C for 10 minutes respectively. Out of the present study findings, it can be concluded that maize cobs are rich source for POD and the applied protocol could be poten-tially used for POD extraction with high level. Meantime, this study suggested a new strategy by which the environ-mental pollution results from accumulation of plant waste can be reduced.

Keywords

aqueous two phase extraction; cetyl trimethyl ammonium bromide; Iraqi maize cobs; maize peroxidase; optimum temperature and pH; thermal stability.

RESEARCH PAPER

An Economical Source for Peroxidase: Maize Cobs☆

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Abstract

According to the current situation of peroxidase (POD), the relevant studies on this enzyme indicated its importance as a tool in clinical biochemistry and different industrial fields. Most of these studies used the fruits and vegetables as source of this enzyme. So that in order to couple the growing requirements for POD with the recent demands for reducing disposal volume by recycling the plant waste, the aim of the present study was to extract POD through management of municipal bio-waste of Iraqi maize species. A simple, green and economical method was used to extract this enzyme. Our results revealed that maize cobs are rich sources of POD, where the activity of this enzyme was found to be 7035.54 U/g of cobs. In pilot experiments this enzyme was extracted from the cobs using an efficient extraction buffer with either Cetyl Trimethyl Ammonium Bromide (CTAB), or sonication. To purify the extracted enzyme the previous step was followed by aqueous two phase extraction (ATPE) using 20% (w/v) polyethylene glycol (PEG) and 9% (w/v) ammonium sulfate. The obtained results indicated that POD was partially purified with 2.36 fold of purification and 81.78% recovery. The optimum temperature and pH of the extracted POD activity as well as the enzyme thermal stability were determined and found to be 20 °C, pH 6, and stable at 60 °C for 10 min respectively. Out of the present study findings, it can be concluded that maize cobs are rich source for POD and the applied protocol could be potentially used for POD extraction with high level. Meantime, this study suggested a new strategy by which the environmental pollution results from accumulation of plant waste can be reduced.

Keywords: Aqueous two phase extraction, Cetyl trimethyl ammonium bromide, Iraqi maize cobs, Maize peroxidase, Optimum temperature and pH, Thermal stability

1. Introduction

Peroxidase (POD) (EC 1.11.1.X) is ubiquitous in microorganisms, plants and animal tissues. It is present in more than one isoform. These isoforms act at different biochemical conditions and subcellular localisation [1–4]. Type III POD is a secretory plant enzyme, which is only localized in vacuoles and cell walls of plants [5,6]. This type plays a vital role in many physiological processes as antioxidant through catalyzing the reduction of H_2O_2 to H_2O [7,8]. Because of its ability to oxidize a broad range of inorganic and organic molecules, it is widely used in clinical biochemistry, environmental, and industrial fields [5,9,10]. Several techniques such as immunohistochemistry [11,12], ELISA [13,14], and treatment of water contamination [15,16] have used this type of POD as a key molecule in these different fields [17]. POD and other enzymes that are isolated for industrial purposes need to be extracted and purified using economic methods that give a high yield.

Enormous amounts of different plant waste are produced during fruits and vegetables consuming, as well as during manufacturing of food products, among these waste are the maize cobs. Maize is considered as a leading product crop throughout the world. It is largely produced by USA followed by China as a main staple food and has been fundamentally consumed by Africa and Latin America [18]. The expanding consumption of maize in schools and colleges, homes, markets and offices etc. implies parallel volumes of its waste accumulation. Such accumulation represents a serious

☆ Special description of the title. (dispensable).

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threat to the human health and negatively affects the environment through producing unacceptable odor and army of flies which normally emanate from heaps of these waste [19,20].

Previously, some studies have obtained high quality and recovery of useful biomolecules from different types of plants such as lipase from germinated seeds of sunflower (*Helianthus annuus*), flaxor linseed (*Linum usitatissimum*), peanut (*Arachis hypogaea*) and castor bean (*Ricinus communis*) [21], total phenolic and flavonoid from *Anacyclus pyrethrm* Link (roots) [22], and POD from Cedar leaf leaves (*Cedrela fissilis*) [23]. Most of these studies used either fruits or vegetables as sources for different biomolecules. Meantime using the edible foods as a source of important industrial molecules seems imprudent practically, unethical conceptually and would lead to raise these natural products prices. On the other hand the massive amount of waste offers significant economic potential for creative uses of these waste other than animal feeds, or fertilizers. Therefore, coupling the requirements for important industrial biomolecules with the recent demands for reducing disposal volume, by recycling natural waste materials (i.e. zero waste) suggests the possibility of trying to utilize these unused plants fractions as a source of important biomolecules. Recently in our laboratory several studies were conducted to extract some biomolecules that have many applications in industry and medicine such as some enzymes [7,24], melatonin [25] and cellulose [26] from different plant waste. In most developing countries there are no obvious strategies for management of maize waste using friendly environment methods. Therefore the present research aimed to investigate the feasibility of using the maize cobs as a cheap and economic source for POD. This was achieved by applying environment friendly method for extraction and partially purification of POD.

2. Materials and methods

2.1. Materials

Acetone, sodium acetate, acetic acid, Cetyl Trimethyl Ammonium Bromide (CTAB), ammonium sulfate $(NH_4)_2SO_4$, polyethylene glycol 6000 (PEG), phenol, 4-aminoantipyrine were purchased from Sigma–Aldrich. Hydrogen peroxide (H_2O_2, 50%) was purchased from local chemist.

2.2. Extraction of crude POD

Cobs of maize (*Zea mays*) (Fig. 1), collected from local markets, were isolated and washed with distilled water then left to dry. These dried cobs were chopped into small pieces using a sharp knife and then it was ground into a fine powder using the blinder. In order to find the most efficient method to extract POD from this part of the maize, several solvents and buffers were tried and as illustrated below and in Fig. 2.

As a first step the powder was treated with acetone at ratio of 1:4 (w/v) and centrifuged at 4000×*g* for 15 min. Then the pellet was treated with acetate buffer 1M, pH6 in a second step. The supernatant of the first and second steps were combined and kept at −20 °C for the next step.

The above mentioned protocol was repeated but with using different buffers each time and as the following: extraction using acetate buffer 0.2M, pH 6 in two steps, extraction using acetate buffer 1M, pH 6 in two steps, extraction using acetate buffer 1M, pH 6 followed by acetate buffer 1M, pH 6 with CTAB (0.5%), extraction using acetate buffer 1M, pH 6 with CTAB (0.5%) in two steps. Finally, the extraction was also done using acetate buffer 1M, pH 6 with sonication in two steps. The crude POD activity and the protein concentration were measured for each sample before storing at −20 °C for the next steps.

Abbreviations

ATPE	aqueous two phase extraction
CTAB	Cetyl Trimethyl Ammonium Bromide
PEG	polyethylene glycol 6000
POD	peroxidase
ppPOD	partially purified POD

2.3. Measurement of POD activity

The activity of POD was measured via continuous spectrophotometric rate determination process according to the method of Song *et al.*, 2005 [27] using hydrogen peroxide as a substrate. A fresh solution of 4-amino-antipyrine (0.0025 M) and phenol (0.17 M) were mixed with 1.5 mL of hydrogen peroxide (0.0017 M) in phosphate buffer 0.2 M, pH 6 then the

Fig. 1. Cobs of Zea mays maize [48].

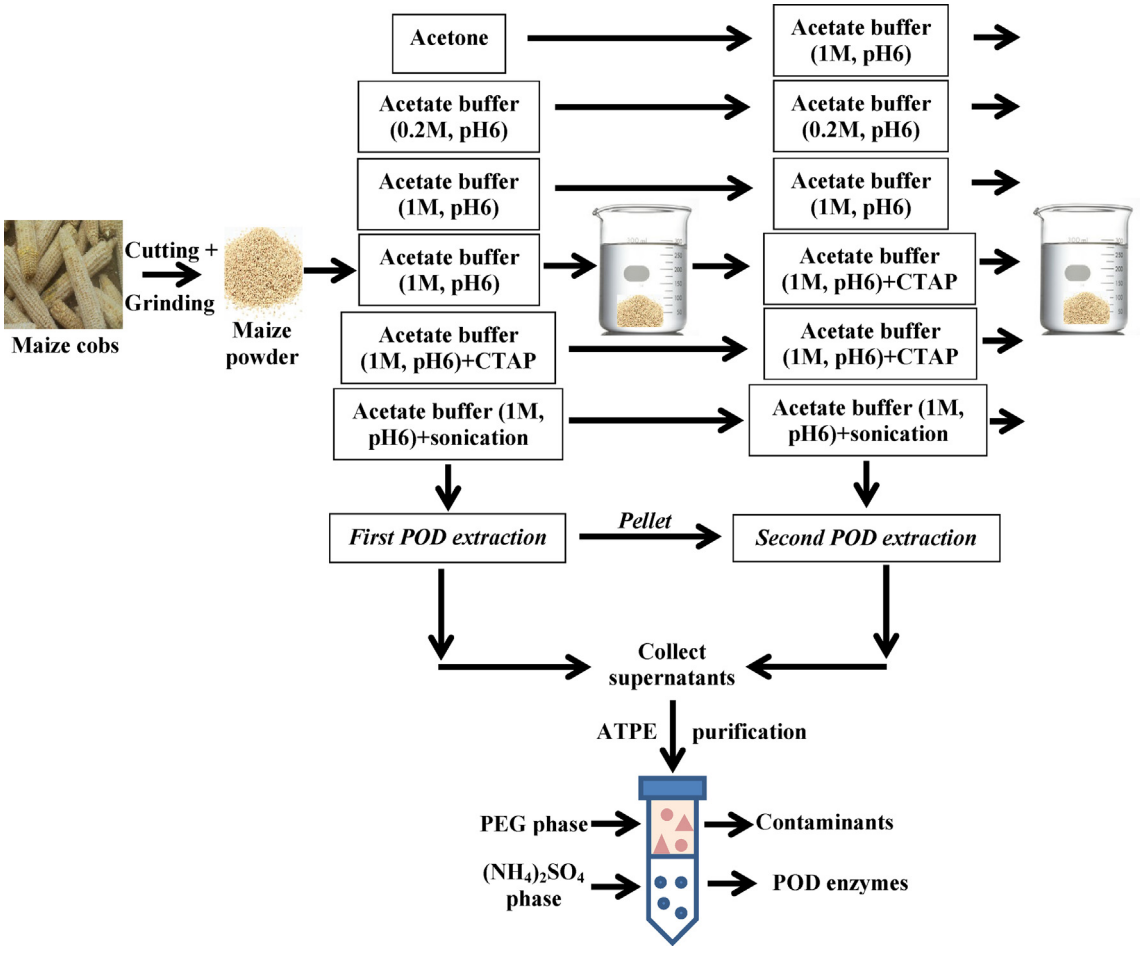

Fig. 2. Optimization of extraction and purification process of POD from maize (Zea mays) cobs.

mixture was incubated for 4 min at 25 °C. The enzyme's extract (0.1 mL) was added to the above mixture. Then the increase in the absorption was followed for 5 min at 510 nm using spectrophotometer (APEL, PD-303, Japan). The POD activity was expressed in unit (U) which was defined as the amount of enzyme that consumed 1 μmol of hydrogen peroxide per minute under the assay conditions. While the specific activity of POD was expressed as a unit of POD activity/g protein. The protein concentration in maize extracts was determined using Bradford method [28] where bovine serum albumin was used as a standard and the measurements of the absorbance were done at 595 nm.

2.4. Partial purification of the POD enzyme

The crude POD extracted from the maize cobs, was partitioned and partially purified using aqueous two phase extraction (ATPE) and as illustrated in Fig. 2. Briefly, Solid PEG 20% (w/v) and solid $(NH_4)_2SO_4$ 9% (w/v) were added to the crude POD extracted from maize cobs, stirred for 1 h then left overnight at room temperature. The mixture was separated into two phases. While only one layer was obtained instead of two layers upon using CTAB, and this one layer mixture was centrifuged at 4000×*g* for 15 min. Then, the POD activity and the protein concentration were measured in aliquot of each sample.

2.5. The optimum pH for POD activity

The optimum pH of partially purified maize cobs POD (ppPOD) activity from maize cobs was determined by monitoring the enzymatic activity at different range of pH (3.0–8.0). The POD extract was incubated with 1M of either acetate, or phosphate buffer using a parallel blank with an appropriate buffer each time.

2.6. The optimum temperature for POD activity

The optimum temperature for the ppPOD activity was determined by assaying the activity using the

Table 1. Comparison between POD activity and specific activity.

Extraction using acetate buffer 1M, pH 6.0 and sonication	Activity (U/mL)	Protein (g/mL)	Specific activity (U/g)
Cobs (Crude)	190577.51	122.42	1556.76
Extraction using acetate buffer 1M, pH 6.0 and CTAB			
Cobs (Crude)	56284.30	8.97	6271.81

optimum pH and at different incubation temperatures (0, 20, 37, 60, 80 °C) for the enzymatic reaction.

2.7. Thermal stability

In order to check the thermal stability of POD, samples of the ppPOD enzyme in its optimum pH were pre-incubated at 60 °C for various times (10, 20, 40 min) in the absence, or presence of $CaCl_2$ (1 mM). Then the enzymatic mixture was cooled in an ice bath to bring the mixture to room temperature. The POD activity and the protein concentration were measured in each case using the above illustrated methods.

3. Results

3.1. Extraction and partial purification of POD

In this study the first tried step was to find the best buffer and protocol to extract POD from cobs part of the maize and as is illustrated in Fig. 2.

As it is obvious from the obtained results Fig. 3, acetate buffer 1M, pH 6 was better to be used in the extraction step than acetate buffer 0.2 M, pH 6. Since the enzymes recovery was obviously increased upon using 1M buffer concentration. Furthermore, using acetate buffer 1M, pH 6 with sonication assisted to recover higher activity of POD. This was also observed when using acetate buffer 1M, pH 6 with CTAB (0.5%). However, POD activity using sonication in the extraction steps was significantly higher than using the same previous buffer including CTAB (0.5%) and without sonication. When a comparison was done between the obtained specific activities of POD that was extracted using these best two methods, the obtained results are presented in Table 1.

It is obvious from these results that the specific activity was higher when the extraction was done in the presence of CTAB (0.5%) compared to sonication. Therefore, the extraction using acetate buffer 1M, pH 6 with CTAP was adopted to provide POD for the next steps of the current study.

The next step was to carry out a pilot experiment to test the possibility of using a green, simple, and economic method to partially purify the extracted POD. This was achieved by using ATPE method with $(NH_4)_2SO_4$ and PEG. The activity and total protein concentration in both crude cob extract and ppPOD samples were measured. The recovery (%) and the fold of purification were calculated and

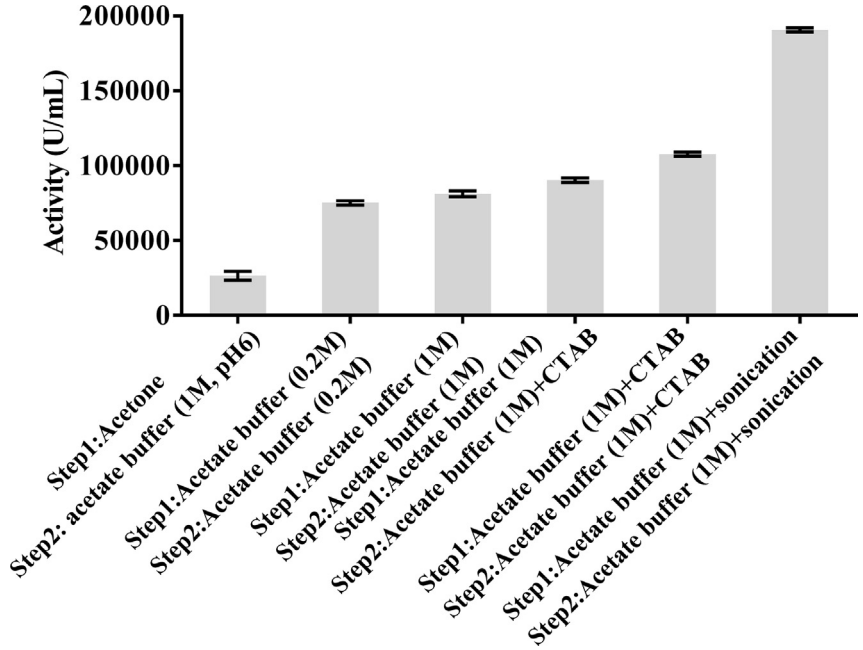

Fig. 3. Activity of POD extracted from maize (Zea mays) cobs using different extraction buffers, where the error bars represent the standard deviation for each mean values.

Table 2. Summary of the partial purification of POD from maize cobs using ATPE method.

ATPE assay + CTAB	POD activity (U/mL)	Protein concentration (g/mL)	POD specific activity (U/g)	Recovery (%)	Fold of purification
Crude Extract	56284.30	8.97	6271.81	____	____
Partially Purified sample	46030.24	3.11	14791.34	81.78	2.36

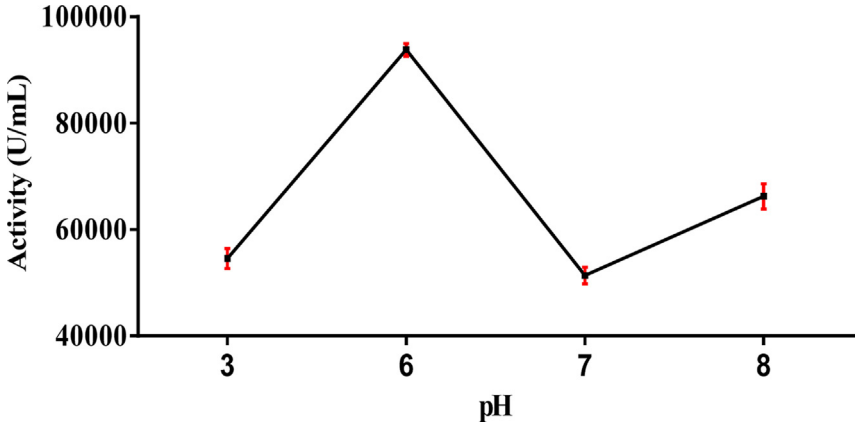

Fig. 4. Optimum pH for the ppPOD extracted from maize (Zea mays) cobs, where the error bars represent the standard deviation for each mean values.

were found to be 81.78% with a 2.36 fold of purification respectively (Table 2).

3.2. Determination of the optimum pH and temperature and the thermal stability of POD activity

The optimum pH for ppPOD activity was 6 as it is clear from Fig. 4. At this pH the enzyme exhibited its maximum in comparison to pH 3 and 7.

Furthermore this enzyme was found to have optimum activity at 20 °C and its activity dropped by 13% at 37 °C and by 67% at 60 °C with no observed activity at 0 °C and 80 °C as the results illustrated in Fig. 5.

The results in Fig. 6 reveal that this enzyme kept all of its activity when heating to 60 °C for a period of 10 min in the absence of calcium ion.While this activity was found to be decreased by 9% and 18% in the absence of this ion after incubation at 60 °C for 20 and 40 min respectively. Meanwhile, it can be observed that the ppPOD was less stable at high temperatures in the presence of calcium ion and the activity was dropped by 10%, 14%, and 8% compared to ppPOD activity in the absence of calcium ion after 10, 20, 40 min of incubation at 60 °C respectively.

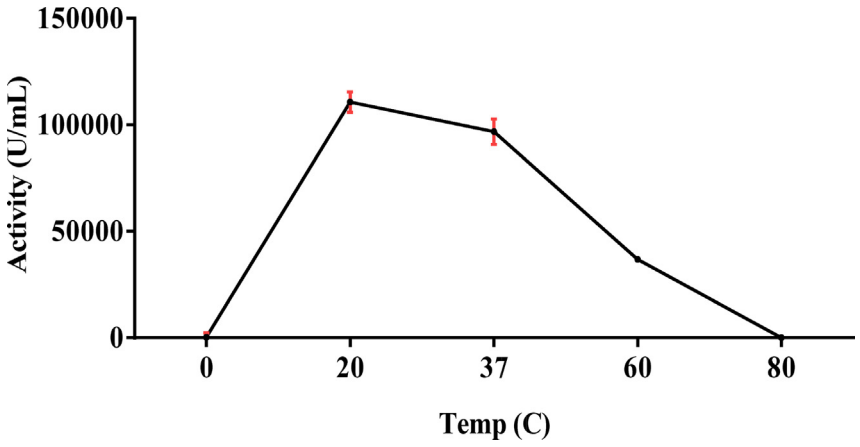

Fig. 5. Optimum temperature study for the ppPOD, where the error bars represent the standard deviation for each mean values.

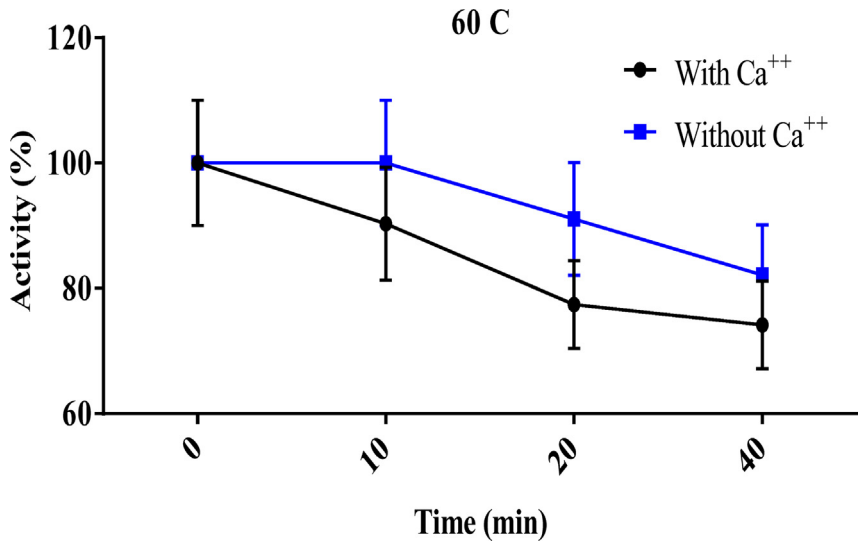

Fig. 6. Thermal stability study for the ppPOD in absence and presence of calcium ions, where the error bars represent the standard deviation for each mean values.

4. Discussion

In the current study we focused on the extraction of POD from municipal maize waste (cobs) using simple and economic method. For the first time, this study showed that POD is present in maize cobs with high activity and specific activity. To the best of our knowledge, no work has been reported world-wide, using this type of plant waste as a source of POD.

Using sonication in the extraction step gave a higher POD activity than using CTAB. In general, sonication is considered as a harsh method for extraction, moreover not all laboratories have the sonicator. Including CTAB, which is a cationic detergent, in the extraction process gave higher specific activity for this enzyme (Table 1). CTAB is considered as a soft detergent that can be used to disrupt, lyse plant cell walls and extract membrane bounds enzymes from these cells. Moreover it is more appropriate for protection the extracted cell proteins including the enzymes from denaturation [29]. The extraction protocol that was used throughout this study indicated that maize cobs are rich source for POD in comparison to other different plant waste listed in the literatures and as it is clear from Table 3. Therefore maize cobs, which is considered as an environment pollutant, can be exploited as an important source for POD enzyme that has many applications [7].

Previously, several studies reported the isolation and purification of POD from different sources using many techniques such as combined more than one chromatography type to obtain the desired purity. Most of these purification techniques require many steps, for example using immobilized metal ion affinity, hydrophobic interaction chromatography and ion exchange chromatography as a major

Table 3. Comparison of POD activity extracted from different plants.

POD source	Scientific name	Activity (U/g of plants)	References
Maize cobs	(*Zea mays*)	7035.54	Our study
Radish	*(Raphanus sativus)*	1100.00	[32]
Turnip	*(Brassica rapa* subsp. *Rapa)*	1500.00	[32]
Cabbage	*(Brassica oleracea)*	900.00	[32]
Tomato	*(Solanum lycopersicum)*	960.00	[32]
Green beans (Outer peel)	(*Vicia faba*)	3.19	[7]
Green beans (Inner peel)	(*Vicia faba*)	0.17	[7]
Green beans (pulp)	(*Vicia faba*)	2.74	[7]
Peas (peel)	(*Pisum sativum*)	6.71	[7]
Watermelon (rind)	(*Citrullus lanatus*)	1.29	[7]
Melon (peel)	(*Cucumis melo*)	8.64	[7]
Bitter orange (peel)	(*Citrus* × *aurantium*)	5.20	[7]
Lemon (peel)	(*Citrus limon*)	8.51	[7]

purification step to get a reasonable result [30]. Moreover they are expensive and not suitable to get the product in a large scale [31]. This led us to look for a low cost method for POD purification.

Using ATPE system has been recommended both in academia and industry as a more reliable for isolation of viruses, membranes, nucleic acids, and different proteins including enzymes from their sources. This system has also been characterized by its selective partition, rapid and simple separation, rapid mass transfer and less denaturation effect with high yield and low cost [33]. Furthermore, it is known that precipitation of proteins by $(NH_4)_2SO_4$ is based on salting out. The main advantage of this process is that it effectively precipitates proteins from biological solution and generally requires a small amount of solvents compared to classical liquid–liquid extraction. It is widely used as an important step in purification of many proteins. Also, PEG, which is a neutral nontoxic water-soluble synthetic polymer, was used as an effective agent in protein fractionation. It is widely used in chemical and biomedical industries, since it has little tendency to denature, or even interact with proteins when it present at high concentrations [34,35].

Applying of ATPE in the purification step gave a high recovery of POD. The fold of purification and the recovery of POD from maize cobs using ATPE were 81.78% and 2.36 respectively. These were compared to other data (Table 4) that obtained using many steps of purification and reported by previous studies.

Table 4 shows that our simple described method gave high recovery with a satisfactory fold of purification in comparison with the used methods in the previous studies [36–38]. And this may be due to losing the enzyme throughout several techniques used by other researchers.

The optimum temperature and pH were studied for the ppPOD as well as its thermal stability. It was found that the optimum pH was 6 with a relatively low activity at pH 8. Similarly, Bania and Mahanta (2012) reported that POD isolated from cabbage and radish showed their highest activity at pH 6 with low activity at pH 8 [39]. Our results are also in good agreement with the values reported by several studies which showed that POD isolated from *Artocarpus lakoocha* [40], the leaves of palm (*Phoenix dactylifera* L.) [41], spinach [42], kiwi [43], and from papaya [44] displayed the highest activity at the pH range 5–12. According to the above mentioned studies, the POD generally exhibits its maximum activity at solutions with neutral to alkaline pH. This may be due to the ionization effects of the acidic pH on the exposed amino acids residues and carbohydrates moiety of enzyme that lead to change the POD interactions with its substrate.

The optimum temperature of the maize POD was found to be 20 °C while only 80% of its maximum activity was observed at 37 °C. These results are in accordance with data that obtained by Yadav et al. (2012) in their study on banana (*Musa paradisiaca*) POD which was 25 °C [45]. In contrast, the optimum temperature for the POD extracted from papaya was reported to have a maximum activity at 40 °C [44] while the optimum temperature of POD extracted from Eupatorium odoratum was reported to be 55 °C [46]. Generally, the discrepancy in value of the optimum temperature and other POD characteristics seems to depend on the source from which the enzyme is extracted.

Our study also showed that the maize cobs POD from maize cobs exhibit high stability at 60 °C for 10 min and it lost about 9% after 20 min and 18% after 40 min of incubation. Surprisingly, it was noticed that POD was less stable at high temperature in the presence of calcium ions where it lost about 10% of its activity compared to its activity in the absence of these ions. These results agree with the result of Omidiji, O et al.(2002) who reported that the presence of calcium ions caused a reduction in POD activity [47]. This might be due to the competition between the calcium ions, as an inhibitor, with the used substrate to bind to the active site of POD. Further kinetic studies are needed to prove our suggestion.

Table 4. Comparison of efficient purification protocols from several studies with our study.

POD Source	Purification steps	Recovery (%)	Fold of purification	References
Maize cobs	1-ATPE	81.78	2.36	Our study
Stem of Arabian balsam	1-Ammonium sulfate precipitation 2- Ion exchange chromatography 3- Gel filtration chromatography	18.50	30.30	[36]
Infected fruit of Solanum sp.	1-Ammonium sulfate precipitation 2-Hydrophobic interaction chromatography 3- Ion exchange chromatography	4.36	1.21	[37]
Plums (*Prunus domestica*)	1-Ammonium sulfate precipitation 2- Ion exchange chromatography 3- Gel filtration chromatography	3.79	26.33	[38]

5. Conclusion

The maize cobs accumulate in huge quantities in the environment as a result of increasing the consumption of maize worldwide which make them as one of environmental pollutant. In order to reduce this type of pollution, the finding of the current study reports and for the first time maize cobs as a rich source of POD. Meanwhile the extraction and partially purification methods that were used in this study have some advantages such as it is green, simple and does not need multiple complicated techniques, therefore it is economic, promising and facile methods for industrial uses.

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