Food Research 7 (4) : 64 - 70 (August 2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Extraction and purification of polyphenol oxidase from edible mushroom (*Agaricus bisporus*) and its use in the manufacture of pastries

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Article history:
Received: 11 December 2021
Received in revised form: 24 February 2022
Accepted: 28 March 2022
Available Online: 3 August 2023

Keywords:
Polyphenol oxidase,
Agaricus,
Edible mushroom,
Pastry

DOI:
https://doi.org/10.26656/fr.2017.7(4).006

Abstract

This study aimed to extract and purify polyphenol oxidase enzyme (PPO) from mushrooms and use it in pastry industry. The enzyme was extracted from edible mushroom using different buffers and purified by several steps. Some Characterizations of the isolated PPO were studied. The activity of PPO was evaluated using spectrophotometric method and catechol as a substrate. Characterization studies indicated that the enzyme showed the highest specific activity 2087 U/mg protein at pH 6.0 using 0.2M phosphate buffer. The ratio of 1:2 (w:v) from mushroom: buffer was the best for obtaining the highest specific activity (2490 U/mg protein). The optimum time of extraction of PPO was 10 mins to obtain high specific activity (2625 U/mg protein). The precipitation of ammonium sulfate and ion-exchange chromatography techniques were performed to purify PPO. The precipitation with saturated solution of ammonium sulfate (70%) was used, the Specific activity was 4332.7 U/mg protein with purification fold of 1.7 and enzymatic yield of 65.5%. Ion exchange chromatography using DEAE cellulose column estimated that the purification times of the enzymatic extract were 6.01, with an enzymatic yield 22.3%. Storage study of the PPO in edible mushroom at 4°C for 3 months indicated that the PPO loss the stability gradually over time, 33% from its original activity retained after 32 days. It can be concluded that edible mushroom is a good source of PPO and the enzyme still active when the mushroom was stored for one month.

1. Introduction

There are many types of mushrooms that are widely consumed as food for their high nutritional value and distinctive flavor as well as medicinal values. Mushrooms have high content of antioxidants and low energy value. They contain vitamins, dietary fibers, proteins, minerals and all the essential amino acids needed by humans (Kalač, 2009). Edible mushrooms also have good contents of biologically active compounds such as ascorbic, alkaloids, carotenoids, flavonoids, folate acid, glycosides, lectins, phenols, terpenoids, tocopherols. These compounds are effective for the prevention of various types of diseases (Kalač, 2013; Valverde *et al*., 2015). The mushrooms have also been used for prevention and treating some diseases such as Parkinson's, Zheimer's, high blood pressure and the risk of stroke, tumor and cancer. In addition, mushrooms contain antimicrobial agents, immune system boosters, and cholesterol-lowering agents (Valverde *et al*., 2015).

Food manufacturers suffer from undesirable coloration in the products that cause problems in the industry, such as the browning of potatoes, eggplant and apples when cutting, and this is undoubtedly considered a major obstacle in the food industry. This effect persists during the storage period and reduces the quality of the food and changes the color, flavor and sensory properties. The food coloring is attributed to one of these enzymatic and non-enzymatic changes. There is another desired or intended change, such as the baking of bread, and this is the result of non-enzymatic browning, such as the caramelization of saccharides and Maillard reactions, and these reactions can be controlled by several factors when used in intended tasks in the food industry (Fang, 2007).

Phenols are found in food, the environment and ubiquitous in nature. These phenols cause food coloration and reduced nutritional and market value. Phenols have antioxidant properties and work against cancer and heart disease. There are three main types of polyphenol oxidase, classified according to the properties of the substrate and their mechanism of action: tyrosinase, catechol oxidase and laccase (ŞimŞek and

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

Yemenicioğlu, 2007; Queiroz *et al*., 2008).

Polyphenol oxidase is a copper enzyme which performs the catalysis of two different reactions that include molecular oxygen as catalyst and phenol. It converts monohydroxyphenols to dihydroxy phenols o-diphenols and then oxidizes to o-quinones and then polymerizes the quinones by the active enzyme cresolasen to form red or black colored pigments (Pandey and Rizvi, 2009; Li *et al*., 2014). Polyphenol oxidase catalyzes the oxidation of phenols, which is responsible for the enzymatic browning in vegetable products and seafood, and the formation of melanin in the skin (Mishra and Gautam, 2016). Tyrosinase represents the main enzyme that is responsible for the enzymatic fruit browning after harvest and the formation of melanin in mammals, an undesirable phenomenon (Fernandes and Kerkar, 2017). This enzyme was named based on the individual substrate it was found to act on like the phenolase, tyrosinase, catecholase, catechol oxidase, diphenol oxidase, cresolase and monophenol oxidase. The structure of the active site of the enzyme includes 2 copper ions, linked by 6 histidine linkages and one cysteine linker. This enzyme is universally distributed in animals, fungi, plants, and bacteria. The abovementioned enzyme is necessary in plants to defend against predators such as herbivores and insects. (Mishra and Gautam, 2016). This enzyme can be intracellularly found in nearly all of the living things, which include animals, plants, and micro-organisms. In the plants, they are involved in the defense mechanism. In the case where the plant is bruised or cut, some of the phenolic compounds are oxidized when the oxygen is present for the formation of a polymeric structure that inhibits microbes (Whitaker, 1996; Alnori *et al.,* 2022).

Because edible mushroom represents a potential source of PPO, this study was conducted in order to extract and purify polyphenol oxidase from it, study some properties of the enzyme and use it in the pastry industry as a browning aid.

2. Materials and methods

2.1 Plant collection

Edible mushroom that has been utilized in the present work was obtained from local market in Baghdad, Iraq. The edible mushroom was washed a number of times by using tap water and then it has been cut and stored at 4°C for further experiments.

2.2 Chemicals

Characterizing PPO has been carried out at chemistry laboratory - College of Agriculture-University of Anbar. All of the solvents and other chemicals were from analytical grade and obtained from Sigma-Aldrich and BDH Chemicals.

2.2.1 Extraction of the enzyme

All extraction steps of PPO have been performed at a temperature of 4^{o}C, 5 g of edible mushroom were homogenized and obtained under conditions of our experiments below.

2.2.2 Determination of polyphenol oxidase activities

Polyphenol oxidase activities have been characterized with using spectrophotometric approach (UV-vis. spectrophotometer, Shimadzu UV-1800, Japan), which is based upon initial increase rate in the absorbance at 410nm at a temperature of 25^{o}C (Soleva *et al*., 2001). The solution of the phosphate buffer pH 7 (0.10M, 1.95mL), 1mL of 0.10M catechol as substrate and 50µL of the extract of the enzyme have been pipetted to a test tube and then vigorously mixed. After that, this mix has been transferred rapidly into 1cm path length cuvette. The activity curves straight-line section as time function has been utilized for the determination of the activity of the enzyme (An enzyme activity unit has been characterized as a change of 0.001 in the value of the absorbance under assay conditions) (Arnnok *et al*., 2010). All the determinations have been carried out in triplicates.

2.2.3 Determination of protein contents

Protein content has been determined in all of the preparations that have been utilized for the PPO assay through calorimetric approach that has been described by. Values have been obtained by the graphic interpolations on calibration standard curve with the bovine serum albumin (BSA) at 595 nm.

2.2.4 Determination of the optimum buffer of extraction

PPO activity has been determined at 3, 4, 5, 6, 7 ,8 and 9 pH values with the use of 0.1 M citrate buffer (pH 3-5), Tris-HCl (pH 8 and 9) and phosphate buffer (pH 6 and 7). time of extraction was 15 mins, and the ratio of extraction was 1:4 (w:v). the enzyme activity and the protein concentration were estimated, and the specific activity was calculated for the determination of optimal time to extract the enzyme.

2.2.5 Determination the best concentration for extraction

Different concentration (0.05, 0.1, 0.15 and 0.2) were tested for the extraction of PPO from edible mushroom using phosphate buffer (pH 6) with 1:4 (w:v) ratio at 15 min for time extraction. The enzyme activity

https://doi.org/10.26656/fr.2017.7(4).006

and concentration of the protein were estimated. the specific activity has been estimated to determine the optimal time for extracting the enzyme.

2.2.6 Determination the optimum ratio of extraction

A total of six extraction ratio including (1:1, 1:2, 1:3, 1:4, 1:5, 1:6) (w:v) were examined to choose the optimum ratio for extraction the PPO from plant using 0.2M phosphate buffer pH 6 and 15 mins. for extraction time. The enzyme activity and concentration of the protein were estimated. The specific activity has been estimated for the determination of the best time to extract the enzyme.

2.2.7 Determination the best time for extraction

Various durations (5, 10, 15, 20, 25 and 30 mins) have been tested for the extraction of PPO from edible mushroom using 0.2 M phosphate buffer and pH 6 with ratio 1:2 (w:v). the enzyme activity and protein concentration were estimated. the specific activity was calculated to determine the best time to extract the enzyme.

2.3. Ammonium sulfate precipitation

The determination of optimum ammonium sulfate concentration for purifications was through treating ammonium sulfate 70% (Gulcin *et al*., 2005). Following the weighing of concentrations of the ammonium sulfate, mixing the enzyme slowly with stirrer over night at 4°C to a point in which it is homogeneous. Afterwards, centrifugation through a speed of 3,500 rpm for 30 mins at 4°C, after completing of the centrifugation process, the precipitate was dissolved in 0.10 M of the Na-phosphate buffer (pH 7) and dialyzed against the sucrose. The activity of the enzyme and the concentration of the protein have been estimated for the solutions.

2.4 Ion exchange chromatography

DEAE-cellulose column was prepared according to a method of (Whitaker, 1972). The dialysate was loaded on the ion exchanger column DEAE cellulose (15×2.5 cm). The separated fractions have been obtained at a 0.50 mL/min flow. The washing step has been accomplished with the use of 0.005 M Tris-HCl buffer (pH 7.2), whereas the step of the elution has been accomplished with the use of the same buffer with sodium chloride salt gradient concentration has been in the range of (0.10-1N). The absorbance of every one of the fractions has been calculated at a 280nm wavelength for the fractions of wash and elution, the activity of the enzyme has been estimated in fractions, after that, the parts of the activation have been severally collected. The activity of the PPO and the concentration of the protein have been estimated (Abdulhakeem *et al*., 2020).

2.5 Storage stability

The enzyme solutions were stored at 4°C for 3 months. PPO activity was measured every 7 days.

2.6 The use of polyphenol oxidase in the pastry industry

A spoonful of yeast and a spoonful of sugar was dissolved in warm water. warm water, half a cup of cooking oil and a teaspoon of salt were added to one kilogram of flour and mixed well, then leaved until it ferments well. Then the dough is distributed in the form of flat balls in a plate, part of it is used as a control, while the other is painted on its surface with different concentrations of the extract (5, 10, 15, 100%) the pastry was prepared based on a traditional procedure with some modification.

3. Results and discussion

3.1 The extraction buffer

Different buffers with different pH and concentration including sodium acetate, potassium phosphate and Tris-HCl were used to determine the best buffer and its concentration for extraction method of enzyme. The results in Figures 1 and 2 showed the differences of PPO activity according to different buffer pH and concentration, the 0.2M phosphate buffer with pH = 6 was the best buffer for extraction in which enzyme activity was 448 U/mL and specific activity was 2087 U/mg protein.

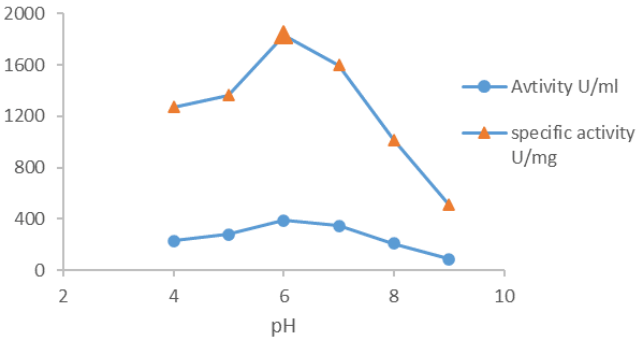

Figure 1. Effect of different buffers on PPO extraction from edible mushroom.

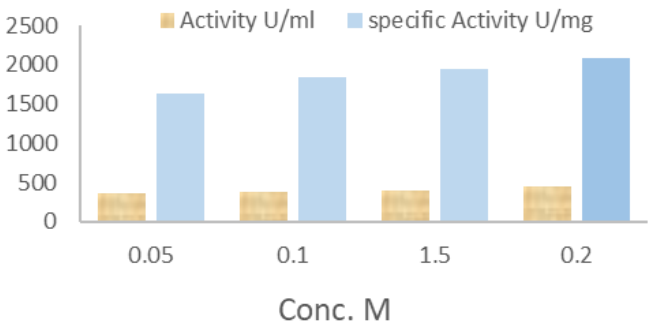

Figure 2. Effect of different concentration of phosphate buffers on PPO extraction from edible mushroom.

https://doi.org/10.26656/fr.2017.7(4).006

From the above result it can be seen that the acidic or amphoteric pH was better in enzyme extraction from edible mushroom, and the differences in values of specific activity belong to effective of nature of buffer and pH because it has the ability to removing the linkages that exist between enzyme and other cellular compounds (Chesworth *et al*., 1998; Hadi and Mariod, 2022).

There are many studies that used different buffers with different pH values for the extraction of the PPO from a variety of sources. The optimum pH value is 7 for Mushroom PPO using catechol as substrate (Zhang and Flurkey, 1997). Maximum activities of the PPO have been observed at pH 4.50 for catechol as substrate (Gulcin *et al.*, 2005). The optimal values of the pH are 6.80 and 5.50 for butter lettuce PPO with the use of 4-methycatechol and catechol as substrates, respectively (Gawlik-Dziki *et al*., 2008). These differences may be attributed to the different sources of mushroom.

3.2 The optimum ratio of polyphenol oxidase extraction

Six ratios were chosen (1:1, 1:2, 1:3, 1:4, 1:5 and 1:6) (w:v) to determine the best ratio of PPO extraction by using phosphate buffer 0.2 M and pH = 6 (Figure 3), the specific activity was measured for crude extract when it recorded the highest value in 1:2 ratio, it was 2490 U/mg protein, followed by 1:1 with 2448 U/mg protein, while the 1:6 ratio gave the lowest specific activity 2016 U/mg protein. When the material-liquid ratio was between 1:3-1:6, the activity and specific activity of polyphenol oxidase decreased. The reason may be that with the increase of material-liquid ratio, the concentration of enzyme was diluted and more hetero-proteins were extracted from mushrooms, leading to the decrease of polyphenol oxidase activity (Mishra and Gautam, 2016). Yanjie *et al*. (2019) found some result (1:2 w/v) for the best extraction ratio of PPO that purified from shiitake mushrooms.

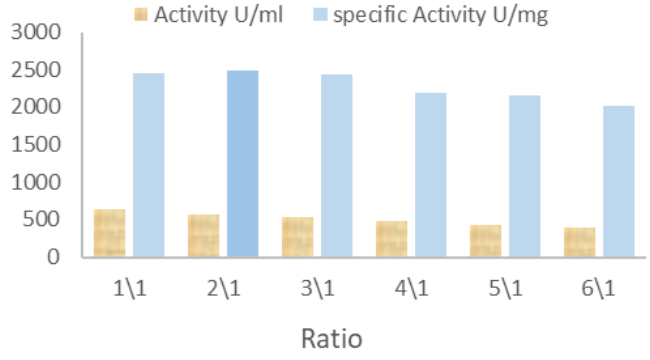

Figure 3. Effect of extraction ratio on PPO activity from edible mushroom.

3.3 Time of extraction

The specific activity of PPO in the extract of phosphate buffer with a concentration of 0.2 M, pH 6 and an extraction ratio 1:2 in different time of extraction (5, 10, 15, 20, 25 and 30 mins.) was estimated to determine the optimal extraction time. The results showed that the optimal extraction time was 10 min (Figure 4). With a specific activity of 2625 U/mg protein. It was noted that the enzymatic activity begins to decrease with time (after 10 mins) due to the hydrolysis of proteins. The degree of degradation depends on the thermal stability of the protein. Determining the optimal extraction time is important due to the difference in the extractability of the enzyme from one source to another due to the diversity of materials present in the source and interfering with the enzyme (Rodwell *et al*., 2016).

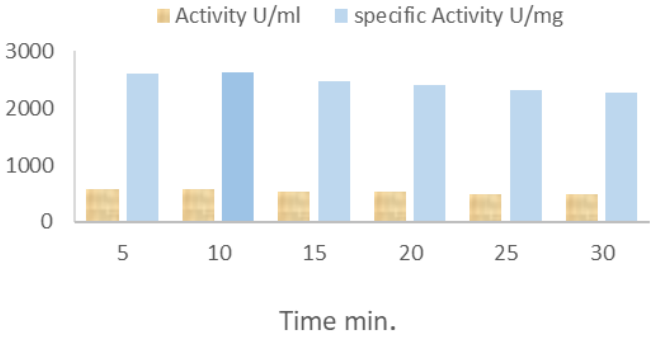

Figure 4. Effect of different extraction time on PPO extraction from edible mushroom.

3.4 Storage stability

The solutions of PPO have been stored at a temperature of 4°C and residual activities have been measured for the estimation of the activity loss throughout the period of storage (Figure 5). The results have indicated that the PPO has been characterized as loss the stability gradually over time. The retained activity is 33% from its original activity over 32 days of storage at 4°C.

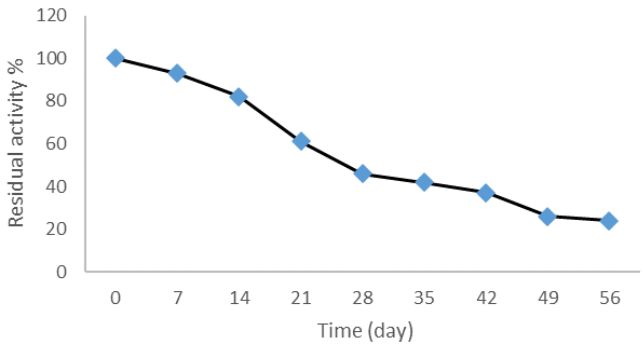

Figure 5. Storage stability of PPO from edible mushroom.

3.5 Partial purification of polyphenol oxidase

A total of two steps was done to purify the PPO

https://doi.org/10.26656/fr.2017.7(4).006

extracted from edible mushroom including precipitate of crude extract by ammonium sulfate and ion exchange chromatography (DEAE-Cellulose). The ammonium sulfate used saturation ratios 70%, the specific activity after dialyzed was 4332.7U/mg, with a 1.70 purification fold, and a 65.50% yield as has been listed in Table 1. Ammonium sulfate has been utilized in the precipitation of the enzyme due to the fact that it is highly soluble and inexpensive in comparison to others salt types, unaffected in the stability of the enzyme and pH. The concentration by the ammonium sulfate based upon equilibrate the charges found in the protein surface and water layer disrupt that surrounds it, which results in its precipitation.

The enzyme solution that has been created from the step of the ammonium sulfate (after being dialyzed) was passed through the DEAE-Cellulose ion exchange column that already equilibrate with Tris-HCl buffer (0.005M, pH = 7.2), the elution step for bonded proteins done when the absorbency reaches the baseline using some buffer with salt gradient (0.1-1 M) NaCl. The absorbance for wash and elution fractions were reading on 280nm. the results shown in appears four protein peaks in the washing step (Figure 6), and only 1 peak of them had shown low activity of the enzyme in the fractions (23-29) so it's ignored. Two peaks had appeared in the step of the elution and only a single peak had shown high activity of the enzyme in the fractions (69-81) these results showed another isozyme of PPO different with pI and charge. the activity fractions in elution step were concentrated. The purification fold of PPO from elution step was 6.01-fold and 22.3% yield (Table 1).

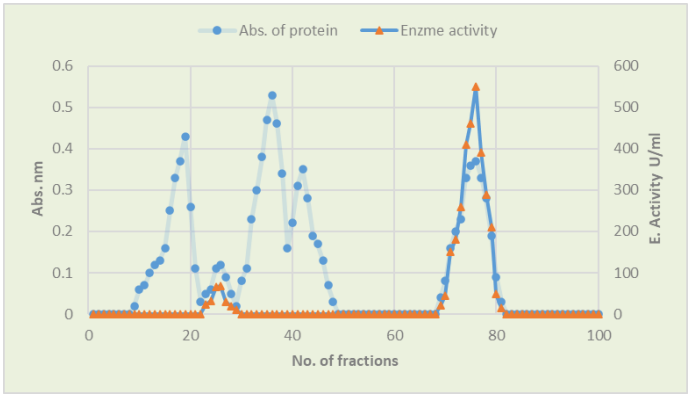

Figure 6. Ion exchange chromatography for purification of PPO extracted from edible mushrooms using DEAE-cellulose column.

Ion exchange chromatography has been utilized in several PPO purification research from plant tissues. had been utilized in the DEAE- cellulose in the purification of the PPO from the Button Mushroom, the fold of the purification has been 16.36 with a yield of 26.6%. PPO from mushroom (*Lactarius piperatus*) has been purified with the use of the Sepharose 4B-L-tyrosine-p-amino benzoic acid affinity column, purification of this preparations was 13.9-fold, some ion exchange chromatography columns used to purification PPO from variety sources. DEAE Sephadex A-50 column used to purification PPO from Seed of Melon (Catherine and Toluwase, 2021) and DEAE-Sepharose to purification PPO from leaves of Cleome gynandra (Gao *et al*., 2011; AL-WARSHAN *et al*.,2023).

The most properties of ion exchange are the matrix (the ion exchanger is made up of an insoluble matrix to which the charged groups were bound covalently. Charged groups have been related to mobile counter ions, which may be exchanged reversibly with other ions of an identical charge with no matrix alteration), Charged groups (The existence of the charged groups has been considered one of the fundamental properties of the ion exchanger. The group type specifies ion exchanger's type and strength; their total amount and availability is a determination of its capacity.

The results of using polyphenol oxidase extract with different concentrations (5, 10, 15, 100%) on the dough showed the occurrence of browning changes of different intensity in the color of the prepared dough. It was observed that the intensity of browning increases with increasing concentration of the extract (Figure 7). This is

Figure 7. Browning changes in the color of the prepared dough using different concentrations (5, 10, 15, 100%) from polyphenol oxidase extract.

Table 1. Purification Steps of PPO extracted from edible mushrooms.

	Volume (mL)	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Total activity (U)	Yield (%)	fold
Crude	15	471	0.185	2545.9	70650	100	1
Ammonium sulfate	19	2435	0.562	4332.7	46265	65.5	1.7
IE	21	750	0.049	15306.1	15750	22.3	6.01

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due to the high percentage of the enzyme in the extract and thus an increase in the enzymatic browning reactions.

Enzyme polyphenol oxidase has been implicated in the reaction of the browning. It oxidises the O-diphenols into the O-quinones, which is a process resulting in the browning reactions in the wounded vegetables, fruits, and plants including apple and garden egg (Adam *et al*., 2016; Hadi *et al.,* 2021). Studies were conducted to compare total phenolic content and polyphenol oxidase (PPO) activities in various milling fractions and determine the role of PPO in flour and dough color. results demonstrated that the browning of dough after 30 mins from dough preparation caused significant decrease in brightness probably due to PPO activity. Therefore, the dough color was more highly correlated with PPO activity.

It has been concluded that the PPO activity levels of the wheat flour is the reason behind color changes of the dough throughout the storage and may be considerably regulated with the flour heat treatment. On the other hand, the effects of the heat treatments on the activity of the PPO have been associated to the treatment approach and duration (Yadav *et al*., 2010).

Conflict of interest

The authors received no specific funding for this work.

Acknowledgments

The authors would like to thank the University of Anbar for support along study period. Also, we thank working staff at College of Applied Sciences, University of Fallujah for the cooperation to do this study.

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