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Production and Purification of Peroxidase from Tissue Cultures of Cauliflower (*Brassica oleracea* L. var. Botrytis)

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Abstract

The plant peroxidases are remarkable enzymes due to their widespread use in industry. These enzymes, which are capable of catalysing the oxidation of various organic and inorganic substrates, have been used in clinical diagnosis, detoxification reactions and organic synthesis. In this study, in vitro production and purification of peroxidase enzyme from cauliflower plant was proposed. Firstly, sterile seedlings were obtained from MS/B5 nutrient medium without growth regulator from cauliflower seeds and calluses from medium containing 0.5 mg / L 2.4-D. Callus and seedlings were powdered with liquid nitrogen and then homogenized. Peroxidase enzymes were purified from these homogenates using affinity technique. SDS-PAGE electrophoresis was performed to determine the molecular weight of the purified enzymes and single bands was observed at approximately 46 kDa. In addition, K_M and V_{max} values of the callus peroxidase enzyme were determined for guaiacol, pyrogallol and H_2O_2 substrates.

Keywords: Cauliflower (b*rassica oleracea* l. var. botrytis), plant tissue culture, peroxidase purification.

1. INTRODUCTION

Enzymes are stable and specific biocatalysts for the processes in which they are used. Enzymes can be used as environmentally sensitive biotechnological approaches in various industrial processes. For this reason, the identification of enzymes and their sources that can be used in industry is of economic and environmental importance [1].

Peroxidases (E.C. 1.11.1.7) are mostly heme-group proteins containing protoporphyrin IX, and their molecular weight ranges from 30 kDa to 150 kDa [2]. It was found that peroxidases play an important role in cell wall formation, auxin metabolism, lignification, removal of reactive oxygen species, fruit ripening and plant defence system [3].

Peroxidases are one of the major enzyme groups used in industrial production and applications. Peroxidases can oxidize many phenolic or non-phenolic substrates, so peroxidase enzymes are frequently used in oxidation reduction based biosensors [4], analytical and diagnostic kits [5, 6], degradation of phenolic compounds [7, 8], organic polymerization reactions [9], paper industry [1] and removal of industrial dyes [10]. In addition, peroxidase applications have been proposed in the medical, chemical and food industries [11]. Some of them relates to the

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quality and flavour of vegetative products in raw and processed foods [12].

Peroxidases are an important part of the world enzyme market. Therefore, they must be produced in large quantities. There are some difficulties in obtaining these enzymes from natural sources. For example, due to the unfavourable weather conditions, the yield is low and the process of growing the plant is long. In order to overcome all these difficulties, plants grown in vitro provides a great advantage to produce significant compounds and molecules [13]. It was aimed to produce peroxidase enzyme continuously by forming in vitro plant cell cultures [14]. The idea that different plant cells can produce more than the peroxidase produced by the roots; such as hairy root of horseradish [15, 16] and *Brassica napus* [17], callus and cell suspension culture of *Raphanus sativus* [18], is becoming more and more common.

In our previous study, peroxidase enzyme (POD) from radish species was successfully purified using Sepharose 4B-L-Tyrosine-4-amino 3-bromo benzohydrazide affinity chromatography [19]. In this study, peroxidase enzymes from cauliflower seedlings (S-POD) and calluses (C-POD) grown with tissue culture method were purified in a single step by Sepharose 4B-L-Tyrosine-4-amino 3-bromo benzohydrazide affinity gel. The purity of enzymes was controlled with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, K_M and V_{max} values of the C-POD were determined for guaiacol, pyrogallol and H_2O_2 substrates.

2. MATERIALS AND METHODS

2.1. Sterilization and Germination

Seeds of *Brassica oleracea* var. botrytis were surface sterilized 70 % EtOH for 1 min, then 20 % NaClO for 20 min, washed 3 times with sterile water. Germination medium was contained MS medium supplemented with B5 vitamins without plant growth regulators and pH of medium was adjusted 5.8 before autoclaving. Seed were germinated at 24±2 ℃ using a 16/8 photoperiod with a light intensity of 50 µmol m^{-2} s^{-1} [16].

2.2. Callus Formation

After 15 days, sterile seedlings were obtained from seeds. The effect of growth regulators was tested using 2.4-D in different concentration (0.5, 1, 2, 3 mg/L) and axillary buds were used as explant from sterile seedlings. The best callus formation was obtained in 0.5 mg/L 2.4-D [18].

2.3. Chemicals

CNBr-Sepharose 4B, L-tyrosine and H_20_2 were obtained from Sigma–Aldrich Co. Methyl 4-amino-3-bromobenzoate from Alfa-Aesar and standard proteins from Thermo Scientific (26616) were purchased. If not explicitly mentioned, all other chemicals used for affinity gel synthesis, purification steps and kinetic determinations are analytical grade.

2.4. Synthesis of Sepharose 4B-based affinity gel

Methyl 4-amino 3-bromo benzoate was purchased and synthesized to benzohydrazide as our previous study described [19]. 1.5 g of CNBr-Sepharose-4B reacted with 30 mg of L-tyrosine to form Sepharose 4B-L-tyrosine. 15 mg of 4-amino-3-bromo benzohydrazide was diazolated and then coupled to the synthesized Sepharose 4B-L-tyrosine. The synthesized affinity gel was formed in three parts; Sepharose-4B (matrix), L-Tyrosine (spacer arm) and 4-amino 3-bromo benzohydrazide (ligand).

2.5. Activity Assay

Peroxidase activity at each step of purification was determined by monitoring the absorbance increase at 470 nm by coloured compounds formed by oxidation of guaiacol chromogenic substrate in the presence of H_2O_2. The reaction medium was formed from 15 mM guaiacol, 7.5 mM H_2O_2, 30 mM phosphate buffer (pH 6.0) and 10 µL enzyme. Activity was defined as the amount of enzyme catalysing the one µmol coloured substance per minute. In kinetic studies with pyrogallol substrate, the method proposed by Bach was used [20].

2.6. Purification of Cauliflower S-POD and C-POD

Seedlings were separated from the stem and cut into small pieces. 5 g of these fractions and calluses were crushed in liquid nitrogen and dissolved in 20 ml of 0.3 M phosphate buffer (pH 7.0). Complete homogenization was achieved with ultraturrax. It was then centrifuged at 15.000g at 4 °C for 20 minutes and pellet discarded. Supernatants were loaded onto the affinity columns (1*10 cm) equilibrated with 10 mM phosphate buffer (pH 6.8). The excess substance was washed with 25 mM phosphate buffer and the peroxidase enzyme was eluted with 1 M NaCl / phosphate buffer. Protein amounts were measured by Bradford method at each step of purification [21].

2.7. SDS-PAGE

SDS-PAGE was performed under denaturing conditions to determine the molecular weight and purity of the purified Cauliflower S-POD and C-POD enzymes. Firstly, samples were loaded to stacking gel (3%) and electric current was applied until the line migrated to 0.5 cm from the bottom of the separation gel (10%) [22]. Then, protein bands were stained with Coomassie dye (R-250). Finally, excess dye was eluted with appropriate solvent and bands were made visible [23].

2.8. Determination of K_M and V_{max} values

The V_{max} and K_M values of the purified Cauliflower C-POD were calculated from the Lineweaver-Burk plots for the guaiacol and pyrogallol and H_2O_2 substrates. Peroxidase activity was measured using 0.1 EU/mL enzyme solution at five different concentrations ranging from 1.8 mM to 32 mM for guaiacol and 0.5 mM to 10 mM for pyrogallol at 470 nm, during which H_2O_2 concentration was kept saturated at 7.5 mM. In order to determine the affinity of C-POD enzyme to H_20_2, activity measurements were made at varied H_20_2 concentration from 0.5 mM to 7.5 mM by keeping guaiacol concentration at 15 mM [24].

2.9. Optimum Parameters and Solvent Stability

For the estimation of optimum parameters and some solvents effected the C-POD activity, enzyme was assayed in varying pHs (4.5 to 9.0), different temperatures (10 to 80 °C) and activity was monitored in varied concentrations of methanol, ethanol and DMSO from 0.1 % to 10 % to investigate the enzyme's solvent stability. At the end of the reactions, all activity values were calculated as relative activity and highest was assigned as 100%.

3. RESULTS AND DISCUSSION

3.1. Purification of Peroxidase Enzymes

One of the important steps in the production of peroxidase is to use techniques such as callus, cell suspension and hairy root cultures which can be produced in a short time under sterile conditions instead of waiting for the product to grow in the field for a year.

As can be seen in detail in Table 1, using the Sepharose 4B-L-Tyrosine-4-amino 3-bromo affinity gel C-POD and S-POD were isolated from the seedlings and calluses of cauliflower with a yield of approximately 30-35% first time. In recent studies, peroxidases were purified using affinity chromatography, purification factors were founded as 665-fold with a yield of 55%, 613- fold with a yield of 34% for turnip and black radish respectively [19]. Using hydrophobic interaction chromatography, horseradish peroxidase was purified with a yield of 71.3% and a factor of 291 [25]. Using three steps, cauliflower peroxidase was purified as a value of 19.3-fold with a yield of 0.2%. The molecular weight of this enzyme was found to be 44 kDa by gel filtration chromatography method [26]. In our study, it is seen that Cauliflower seedlings and calluses have more enzymes than native cauliflower and tissue culture is advantageous in the production of peroxidase.

POD produced by Red beet (*Beta vulgaris* L.) hairy root was purified with 15-fold in a cell culture study and also the purified POD had a

molecular mass of 45 kDa as predicted by SDS-PAGE in the same research [27]. *Agrobacterium rhizogenes* transformed *Armoracia rusticana* L. tissue cultures of transformed roots were found to have 20 times higher activity than native horseradish plant [16]. In another study, peroxidase activity was found to be high in cell-free medium in cell suspension cultures of *Raphanus sativus*. In addition, it has been reported that *Raphanus sativus* cell suspension cultures can produce peroxidase production as an alternative to horseradish roots [18]. It is supported by studies carried out in different plant groups where peroxidase can be produced and purified in different cultures in biotechnologically.

Table 1 Purification results of S-POD and C-POD from cauliflower seedling and callus by affinity chromatography

Source	STEP	T.V.[a] (mL)	Activity (EU/mL)	Protein (mg/mL)	T.A.[b] (EU)	T.P.[c] (mg)	S.A.[d] (EU/mg)	Yield (%)	Fold
Seedling	**I**	6	20.3	1.19	121.8	7.14	17.06	100	1
	II	1.5	23.8	0.0011	35.7	0.0017	21000	29.3	1230
Callus	**I**	6	30.6	1.57	183.6	9.42	19.5	100	1
	II	3	21.5	0.0015	64.5	0.0045	14300	35.1	734

Total volume[a], Total activity[b], Total protein[c], Specific activity[d] Step I: Homogenate Step II: Purification with affinity chromatography

Cauliflower peroxidase, which is considered as an alternative to horseradish peroxidase, was rapidly propagated by tissue culture methods and this enzyme was purified from the obtained seedlings and calluses. According to this, 30% production of seedlings and 35% (Table 1.) production of calluses occurred. This study is one of the new-first studies that will be an example of peroxidase enzyme purified from seedling and callus growing by tissue culture methods in cauliflower plant.

3.2. SDS-PAGE

SDS-PAGE was performed to determine the purity and molecular weight of C-POD and S-POD enzymes isolated from cauliflower. The molecular weight (Mw) of these enzymes were found to be single bands at 46 kDa as shown in Figure 1.

Koksal and Gülçin determined the molecular weight of the purified POD enzyme from cauliflower to be 44 kDa [26]. Similarly, the molecular weight of the POD enzyme produced by the hairy root of beet (*Beta vulgaris* L.) was found to be 45 kDa by SDS-PAGE [27]. The fact that these values are consistent with the data in the literature is an indication of the successful purification of enzymes.

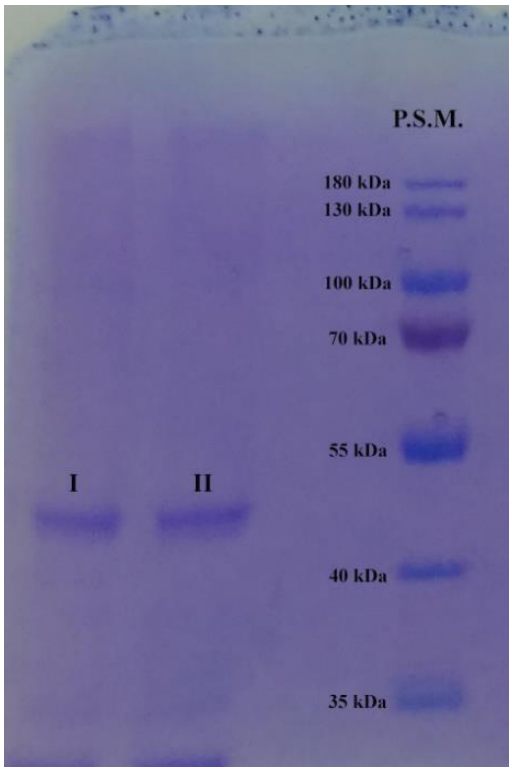

Figure 1 SDS-PAGE image of purified S-POD and C-POD enzyme. **P.S.M: Prestained protein markers, *I: S-POD, *II: C-POD*

3.3. Determination of K_M and V_{max} values

Determining the kinetic parameters of the purified peroxidase enzyme in the presence of different substrates is one of the important data that should be obtained. These data help to determine the physiological role of the enzyme and whether it

can be used in vitro applications. For this reason, activity-concentration graphs were plotted for guaiacol, pyrogallol and H_2O_2 substrates. Activity-concentration graphs were shown in Figure 2. K_M-V_{max} values were calculated from these graphs for C-POD enzyme as 9.34 mM and 0.36 EU/mL.min, 1.8 mM and 0.3 EU/mL.min, 0.98 mM and 0.19 EU/mL.min respectively.

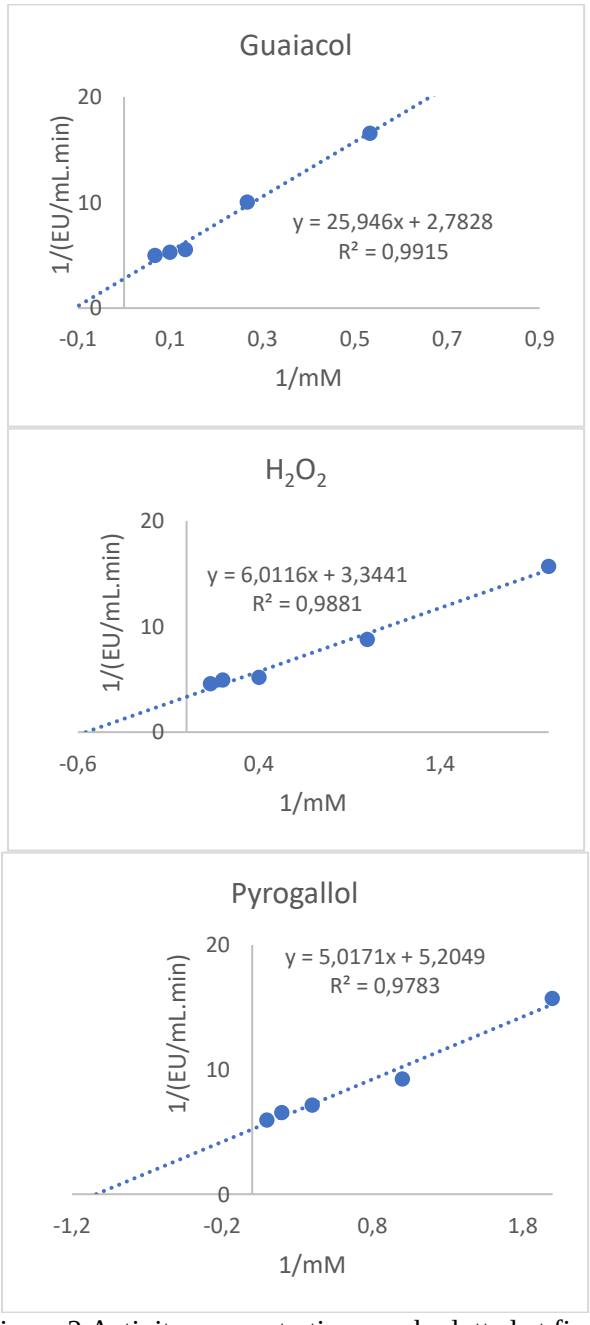

Figure 2 Activity-concentration graph plotted at five different guaiacol, H_2O_2 and pyrogallol concentrations

In a study with native cauliflower peroxidase K_M affinity of POD for guaiacol and pyrogallol were determined as 141.64 mM and 1.1 mM respectively. [26]. In another study, K_M affinity and V_{max} values of *Beta vulgaris* L peroxidase for guaiacol were 98.61 mM and 0.107 EU/mL min, for orthodianisidine were 2.134 mM and 0.977 EU/mL min., for ABTS were 5.050 mM and 0.110 EU/mL , for H_2O_2 were 0.113 mM and 1.389 EU/mL min [27].

When comparing the data in the literature and the data we obtained in our study, the substrate specificities of the peroxidase enzymes purified from different sources or from different regions of the same sources vary. Therefore, especially K_M value is an important indicator in determining whether the enzyme can be used in applications.

3.4. Temperature, pH and Solvent Effects on Activity

The effects of temperature, pH and solvents on catalyst capacity of the enzyme were plotted as activity graphs (Figure 3). In obtained results, the maximum catalysing capacity of C-POD was observed at 60°C and pH 5.5. Moreover, the relative activity was recorded at 82%, 69% and 45% in the presence of %10 (w/w) methanol, ethanol and DMSO, respectively. It can be concluded that it is a great advantage that the C-POD enzyme obtained for the catalysis of reactions containing water-insoluble organic substrates is not affected much by the presence of these solvents (especially alcohols), which are frequently used in industrial processes.

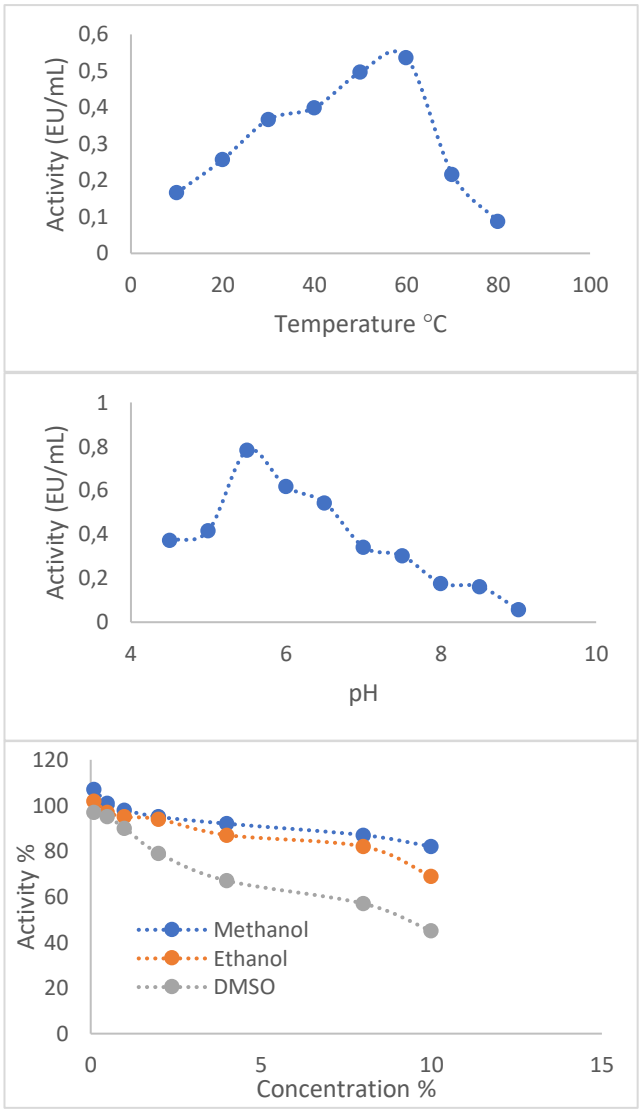

Figure 3 Activity graph plotted temperature, pH and solvents on catalyst capacity

4. CONCLUSION

Peroxidases are important commercial enzymes. Therefore, there is a need to produce and purify peroxidases which can respond to different needs and are resistant to factors such as temperature, pH, salts, metals and organic solvents in the reaction medium. In this study, peroxidase enzymes were obtained from cauliflower seedlings and calluses for the first time. In addition, molecular weights of C-POD and S-POD enzymes and affinity of C-POD to H_20_2, pyrogallol and guaiacol substrates were determined. Cauliflower plant callus can be easily modified, disease-free and sterile conditions; it is thought that large amounts of commercially important peroxidase production can be realized by experimenting with different nutrient media and growth regulators.

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

Authors' Contribution

Under this heading, The authors contributed equally to the study

The Declaration of Ethics Committee Approval

This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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