Purification and selected biochemical properties of peroxidase from cress (Lepidium sativum sub sp. sativum)

Ahmet Altay\(^a\), Tubanur Koktepe\(^a\), Lokman Durmaz\(^b\), Fevzi Topal\(^c\), İlhami Gülçin \(^d\), and Ekrem Köksal\(^a\)

\(^a\)Faculty of Sciences and Arts, Department of Chemistry, Erzincan University, Erzincan, Turkey; \(^b\)Cayırlı Vocational School, Department of Medical Services and Technology, Erzincan University, Erzincan, Turkey; \(^c\)Gümüşhane Vocational School, Department of Chemical and Chemical Processing Technologies, Laboratory Technology Program, Gümüşhane University, Gümüşhane, Turkey; \(^d\)Department of Chemistry, Faculty of Sciences, Atatürk University, Erzurum, Turkey

**ABSTRACT**

Cress (Lepidium sativum) is an annual herb from Brassicaceae family, and in some regions. It is known as peppergrass, garden cress, garden pepper-cress, pepperwort, or poor man’s pepper. Cress is an important medicinal plant in some countries including Turkey. This plant has major scientific and therapeutic significance. Peroxidase (POD, E.C.1.11.1.7) is an oxidoreductase enzyme produced by a number of organisms. In this study, POD enzyme was purified from cress by ammonium sulphate precipitation, gel filtration, and CM-Sephadex ion-exchange chromatographies. K\(_m\) and V\(_{\text{max}}\) values were calculated from the Lineweaver-Burk graph for H\(_2\)O\(_2\) and guaiacol substrates and the substrate specificity of POD was investigated. The results showed that substrate specificity of H\(_2\)O\(_2\) is better than substrate specificity of guaiacol for this enzyme. The inhibition effects of three cations (Al\(^{3+}\), Cu\(^{2+}\), and Cr\(^{3+}\)) and one organic compound of cetyl trimethylammonium bromide were performed and their inhibition types were determined. Finally, optimum pH, optimum temperature, optimum ionic strength, and stable pH were determined.

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**CONTACT** İlhami Gülçin igulcin@atauni.edu.tr
Department of Chemistry, Faculty of Sciences, Atatürk University, Erzurum, Turkey

**INTRODUCTION**

Medicinal plants have long been utilized since the advent of human civilization.\(^{[1]}\) The use of these plants for their medicinal properties is a very old practice. Their origins in the oldest civilizations are still well-preserved for many centuries all over the world.\(^{[2]}\) They are used by millions of people worldwide, not only by rural inhabitants, but also increasingly by urban citizens in both developing and developed countries.\(^{[3-5]}\) Cress (Lepidium sativum) is an annual herb from Brassicaceae family and, in some regions, is known as garden cress, garden pepper cress, pepper grass, pepperwort, or poor man’s pepper. Cress is an important medicinal plant in some countries. This plant has a major scientific and therapeutic significance. It can be grown at all elevations, throughout the year, but the best crop is obtained in the winter season.\(^{[6]}\) It is a plant that can grow faster and is a steep, annual, herbaceous plant, which grows up to 15–45 cm height and belongs to the mustard family. The plant has many branches on the upper part, and seeds, leaves, and roots of cress are economically valuable in some countries. Their leaves are generally used in salads and soups.\(^{[7]}\)

Peroxidase (POD, E.C.1.11.1.7) is one of the primary enzymes responsible for browning process\(^{[8]}\) and commonly found in plants.\(^{[9,10]}\) POD can promote darkening in plants, although it is limited by the...
availability of electron acceptor compounds such as superoxide radicals, hydrogen peroxide, and lipid peroxides. On the other hand, lactoperoxidase is the most known POD and a member of the haeme POD family. Lactoperoxidase is secreted from salivary, mammary, and other mucosal glands, which had functions as a natural antibacterial agent. Therefore, the inhibition of POD is important for better colour retention and for increasing shelf-life. Hydrogen peroxide ($H_2O_2$) has harmful potential for cell walls and POD is a basic system for removing $H_2O_2$, in which excessive production of $H_2O_2$ can have hazardous effects for antioxidant balance in living organisms. This compound is naturally produced in livings as a by-product of oxygen metabolism. Almost all organisms contain POD enzymes, which harmlessly and catalytically decompose low concentrations of $H_2O_2$. Hydrogen peroxide plays an important role as a signalling molecule in the regulation of a wide range of biological processes. POD has been purified from many different sources like wheat, spinach, and bean. Some of their biochemical properties including thermal stability, optimum and stable pH, optimum temperature, and enzyme kinetics have been determined. In general, similar methods like ammonium sulphate precipitation, dialysis, ion exchange, and gel filtration chromatographies have been used for purification studies, especially made from plant sources.

In the present study, the enzymatic properties of Cress (Lepidium sativum) POD (cPOD) were studied from a new source. cPOD was purified using ammonium sulphate precipitation, gel filtration chromatography and ion exchange chromatography. The purity of the enzyme was checked using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Also, optimal conditions, thermal stability, and kinetic and inhibition properties of cPOD were performed.

Materials and methods

Plant materials and chemicals

The plant materials were provided from a local market in Erzincan, Turkey. Cress (Lepidium sativum) sample was identified by Dr. Ali Kandemir from Biology Department, Erzincan University. Plant materials were deposited in Erzincan University Herbarium (Herbarium no: Kandemir-10961). Then, the aerial parts of the plant materials were washed, dried, and placed in a polyethylene bag and stored in the refrigerator until the start of enzyme extraction. The chemicals used in the studies were supplied from Merck and Fluka.

Preparation of the plant extract

A portion of cress (50 g) was taken from the refrigerator and left in a mortar. Then, liquid nitrogen was added to the plants and crushed to powder form. The powder of cress was mixed with 100 mL of phosphate buffer (0.1 M, pH 7.0) and was centrifuged at 12000 x g for 60 min at 4°C. The pellet was eliminated. The sediment was suspended in about 3 mL phosphate buffers and dialysed for 10 h against phosphate buffer (10 mM, pH 7.0). This sample was used for other parts of the study.

Protein precipitation with neutral salts

For the first purification step, powdered cress sample (20 g) was suspended in phosphate buffer (0.1 M, pH 7.5) and the homogenate was incubated at 20°C for 30 min. After removing the debris, the sample was centrifuged at 12000 x g for 20 min at 4°C. The pellet was discarded and the supernatant was collected and referred to as sample extract. Finely ground weighed ammonium sulphate was added to the sample extract with constant stirring in an ice bath. Fractionation of proteins involved of 0–10% to 70–80% saturation with ammonium sulphate after each saturation step. Sample extract was centrifuged at 10000 x g for 60 min. The supernatant was kept and the resulting pellet was dissolved in 2 mL phosphate buffers (0.1 M, pH 7.0). The fraction with maximum POD activity was chosen and dialysed in a dialysis tube against phosphate buffer (0.1 M, pH 7.0) for 24 h with NaCl (1.0 mM).
**Determination of POD activity**

The POD activity in cress sample was determined using guaiacol and H$_2$O$_2$ as the substrates. This activity assay was based on the measurement of tetra guaiacol formation in the presence of guaiacol and H$_2$O$_2$ within 3 min. The temperature of the mixture was maintained by a water bath at 25°C. The final mixture contained 50 µL enzyme sample, 45 mM guaiacol (1.0 mL), 22.5 mM H$_2$O$_2$ (1.0 mL), and 950 µL phosphate buffer (0.1 M, pH 7.0).

**Qualitative and quantitative protein determination**

Bradford method was used for determination of the protein quantity. On the other hand, the qualitative protein amount was measured at 595 nm. In this method, the complex forms between proteins and Coomassie Brilliant Blue G-250 agent in the presence of phosphoric acid. Bovine serum albumin is used as a standard protein.

**Preparation of CM-Sephadex A-50 ion exchange chromatography**

Seven grams of CM-Sephadex A-50 was mixed with 100 mL deionized water and left in 90 °C for 6 h. On cooling to the room temperature, 100 mL NaOH (0.5 N) was added to this mixture and was allowed to stand for 1 h. Then, the supernatant was decanted and the exchanger was washed with deionized water until the effluent had neutral pH. Afterwards, the exchanger was stirred in 100 mL HCl (0.5 M). Subsequently, the CM-Sephadex A-50 ion exchange chromatography column materials were washed with deionized water until the effluent reached pH 7.0. Finally, the exchanger was suspended in 0.1 M phosphate buffer (pH 8.5) and was then packed in a column (2.5 x 30 cm), washed, and equilibrated.

**Purification of peroxidase by cation exchange chromatography**

Briefly, the column was packed with CM-Sephadex A-50. It was equilibrated with 1.0 L of Tris-HCl buffer (0.1 M, pH 8.5). Then, the enzyme homogenate was loaded onto the column and washed with equilibrating buffer. Proteins were eluted with a gradient of 0–1 M NaCl in phosphate buffer (10 mM) at a flow rate of 15 mL/h by using a gradient mixer apparatus. The fractions were collected and absorbance of each fraction was spectrophotometrically recorded at 280 nm. cPOD activity was measured at 470 nm for fractions, which showed absorbance at 280 nm.

**Gel filtration chromatography**

The fractions showing activity from the ion exchange chromatography were pooled and applied on a Sephadex G-100 equilibrated with phosphate buffer (6 mM, pH 7.0) at 4°C. cPOD was again eluted with a linear 0–100 mM NaCl gradient at a flow rate of 15 mL/h. Then, the absorbance of protein in collected fractions was spectrophotometrically measured at 280 nm. On the other hand, the cPOD activity was determined at 470 nm.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

In order to check the purity of the enzyme and determine its molecular weight (MW), the pooled enzyme from gel filtration chromatography was applied to SDS-PAGE according to Laemmli’s procedure as described previously in detail. Discontinuous electrophoresis was conducted by using 12.5% and 4% stacking gel. The gel was stained in Coomassie Blue and afterwards it was washed.
**Selected biochemical properties**

**Effect of temperature on cPOD activity**
cPOD activity was determined at different temperatures. At a certain temperature, the enzyme activity was measured by the addition of enzyme to the mixture as rapidly as possible. The process was performed by a circulatory water bath in a temperature range between 0°C and 80°C.

**Effect of pH on cPOD activity**
cPOD activity was determined in the range of pH 3–9 using the following buffers: 0.01 M acetate buffer, pH 3.0–4.5, and 0.01 M phosphate buffer, pH 5.0–7.5, 0.01 M Tris/HCl buffer, pH 8.0–9.0. For all measurements, H₂O₂ and guaiacol were used as substrates.

**Stable pH of cPOD**
cPOD activity was observed for 18 days to determine the stable pH value of the cPOD. The process was carried out using three different buffers with pH range between pH 3.0 and pH 9.0 at 1.0 pH intervals.

**Effect of ionic strength on cPOD activity**
For determination of ionic strength on cPOD activity, different concentrations of phosphate buffers (0.025–0.25 M) were used.

**Kinetic properties of the cPOD**
Kₘ and Vₘₐₓ values of cPOD were determined for guaiacol and H₂O₂ substrates. cPOD activity was measured using five different substrate concentrations. Kₘ and Vₘₐₓ values were calculated from Lineweaver-Burk graphs. Then, the Vₘₐₓ/Kₘ ratio was calculated and catalytic power value was determined for each substrate and the value of this ratio determines the more effective substrate.

**In vitro inhibition assay**
The in vitro inhibition effects of different inhibitors were studied against cPOD. The inhibitors were prepared at different concentrations. Then, the final solution mixed at room temperature and their activity was spectrophotometrically measured. The IC₅₀ values were calculated from activity (%) versus inhibitor concentration plots. Diverse substrates (guaiacol and H₂O₂) concentrations were used and Lineweaver–Burk graphs were drawn to obtain the Ki constant of inhibitors.

**Results and discussion**

**Chromatography studies**
In this study, two different chromatographic methods were used to purify POD from fresh cress plant. Before chromatography processes, ammonium sulphate precipitation was performed in order to decrease the amount of other proteins. Ammonium sulphate was used as salt due to its high resolution in water. Then, the precipitated proteins were dialysed against phosphate buffer (1.0 M and pH 7.0) (Figure 1a). This homogenization was applied directly to gel filtration chromatography for further purification steps. The fractions (approximately 3 mL) were collected using by neutral salt gradient including NaCl (1.5 M) and phosphate (0.1 M). For each fraction, the quantity of protein was spectrophotometrically measured at 280 nm. Then, the absorbances of fractions were measured at 470 nm (Figure 1b). The fractions, which possess cPOD activity, were pooled and applied to CM-Sephadex cation exchange chromatography column (Figure 1c). As a result of this process, cPOD was purified 46.9-fold with 0.61% yield (Table 1). Similarly, POD was purified from *Aspergillus terreus* 13.1-fold with a yield of 14.2%. In another study, POD was purified from *Ficus carica* by ion exchange on CM-
Sepharose and DEAE-Sepharose columns followed by gel filtration on Sephacryl S-200 column chromatographies with purification folds of 3.05 and 0.79 and recovery percentages of 50.2% and 11.32%, respectively.\[59\] On the other hand, POD obtained from Euphorbia tirucalli was purified using by CM-Sepharose and resulted in 2-fold purification and 12% recovery.\[60\] Similarly, POD obtained from Citrus jambhiri was purified using by CM-Cellulose column and resulted in 13.10-fold purification and 9.7% recovery.\[61\]

**Determination of molecular weight and checking of enzyme purity**

For checking of enzyme purity and its MW of cPOD, SDS-PAGE was performed.\[62\] PODs are divided into two major families including plant and animal enzymes with MWs range between 30 and 150 kDa.\[59\] Electrophoretic results of these studies are given in Figure 2. The single band showed that purification studies were successful and MW of cPOD was 44.02 kDa. This result is consistent with many previous studies. For example, MWs of POD purified from different plants have been reported for Aspergillus terreus as 43 kDa,\[58\] for cauliflower (Brassica oleracea L. var.

**Table 1.** Purification steps of peroxidase from cress (Lepidium sativum sub sp. sativum).

| Purification steps | Total volume (mL) | Enzyme activity (EU/mL) | Total enzyme activity (EU) | Protein (mg) | Total protein (mg) | Specific activity (EU/mg protein) | Yield (%) | Purification fold |
|--------------------|-------------------|-------------------------|---------------------------|--------------|-------------------|-----------------------------------|-----------|------------------|
| Homogenate         | 50                | 1038                    | 51900                     | 1.34         | 67.0              | 774.63                            | 100.0     | 1.0              |
| (NH₄)₂SO₄ precipitation | 12               | 1573                    | 18876                     | 1.066        | 12.8              | 1474.68                           | 36.4      | 1.9              |
| Gel filtration chromatography | 3                | 708                     | 2124                      | 0.170        | 0.51              | 4164.70                           | 4.1       | 5.4              |
| Ion Exchange chromatography | 3                | 104                     | 316                       | 0.0029       | 0.0087            | 36321.83                          | 0.61      | 46.9             |

Figure 1. Purification of peroxidase (cPOD) from cress (Lepidium sativum sub sp. sativum). (A) Ammonium sulphate precipitation, (B) Gel filtration chromatography, (C) CM-Sephadex ion exchange chromatography.
botrytis) buds as 44 kDa,\(^{[53]}\) and for haricot bean (\textit{Phaseolus vulgaris}) as 45 kDa.\(^{[33]}\) Also, a denatured POD from \textit{Euphorbia tirucalli}, which migrated in SDS-PAGE, had a single band with a MW of 44 kDa.\(^{[60]}\) The differences in the MW of plant POD could be due to differences in amino acid sequences depending on glycosylation levels.\(^{[57]}\) In a recent study, POD from \textit{Ficus carica} was found as monomeric with a MW of 30 kDa.\(^{[59]}\) In addition, POD from \textit{Ficus sycomorus} had a molecular mass of 43 kDa.\(^{[60]}\) In contrast, POD from \textit{Leucaena leucocephala} had a heterotrimeric structure consisting of two subunits of 66 kDa and one subunit of 58 kDa.\(^{[63]}\)

**Characterization studies**

In order to determine the characterization of cPOD, enzyme activity was measured at different pH, temperature, and salt concentrations. As shown in Figure 3a, thermal stability of cPOD was examined at temperatures ranging from 10° to 70°C. The optimum temperature for cPOD activity was found to be 50°C; however, the enzyme activity was stable from 20°C to 40°C. The enzyme activity was rapidly decreased with the rise of temperature. A wide variability concerning the optimum temperature for POD activity had been reported from various sources including Caribbean plant (50°C),\(^{[64]}\) \textit{Allium sativum} (84°C),\(^{[65]}\) and vanilla bean (16°C).\(^{[66]}\) The enzyme activity against temperature resistance depends on the source of the enzyme as well as on the assay conditions, especially pH and the nature of the employed substrate. The variability in the heat stability of POD can be attributed largely to the particular enzyme structure.\(^{[67]}\)

In this study, it was shown that cPOD is more active in acidic conditions. The effect of pH on the activity of cPOD is demonstrated in Figure 3b. The optimum pH of cPOD was determined as 6.0 using 0.1 M sodium phosphate buffers. The results of the salt concentration study revealed that the effects of the salt concentration are low and similar results have been found for different salt concentrations. The optimum pH of POD from \textit{P. chrysosporium}, \textit{P. sordida}, \textit{D. squalens}, and \textit{L.}}
The optimum pH can be different depending in various sources like POD from pineapple (4.2), banana (4.5–5.0), horseradish peroxidase (4.6–5.8), potato (5.0–5.4), garlic (5.0), garlic (5.0), and fingerroot (6.0). [58] [69]

cPOD activity was determined at 63 mM of salt concentration as the maximum value (Figure 3c). To estimate the stable pH value of cPOD, the activity was monitored in three different buffers and in seven different pH values for 18 days. It was demonstrated that cPOD was more stable in neutral conditions (Figure 3d). However, the most stable pH value for cPOD was found to be pH 7.0 in neutral acidity. Also, it was reported that the POD from Aspergillus terreus was stable in the pH range from 11 to 12.5 with the optimum pH being 12.5. These results showed that optimal and stable pHs range of the POD from Aspergillus terreus are in the alkaline range. [58]

**Kinetic studies**

To be able to determine the substrate specificity of the enzyme to guaiacol and hydrogen peroxide substrates, $K_m$ and $V_{max}$ values were determined for guaiacol/H$_2$O$_2$ substrate pairs. To this end, the enzyme activities were measured at five different concentrations of hydrogen peroxide, while guaiacol concentration was constant. This process was repeated for H$_2$O$_2$, while guaiacol concentration was constant. After this process, Lineweaver-Burk graphs were drawn and $K_m$ and $V_{max}$ values were calculated from these graphs (Figure 4a–b). The enzyme has $K_m$ values of 28.79 and 0.73 mM for guaiacol/H$_2$O$_2$ substrate pattern, respectively. On the other hand, the enzyme has $V_{max}$ values of 1217.1 and 727.11 EU/mL.min for each substrate, respectively. Hydrogen peroxide has a higher $V_{max}/K_m$ ratio and therefore it is a more effective substrate than guaiacol.
Inhibition properties of the enzyme

The effects of three metal ions and an organic compound of cetyl trimethylammonium bromide (CTAB) on cPOD activity are summarized in Table 2. While CTAB effectively inhibited the cPOD activity, metal ions including Al^{3+}, Cr^{3+}, and Cu^{2+} demonstrated moderate inhibition effects against

Table 2. Inhibition properties of some inhibitors on cPOD activity.

| Metal ions | Concentration (mM) | IC_{50} (mM) | Ki (mM) | Inhibition type       |
|------------|--------------------|--------------|---------|-----------------------|
| Al^{3+}    | 0–30              | 8.36         | 5.78    | Noncompetitive        |
| Cu^{2+}    | 0–30              | 31.56        | 20.90   | Noncompetitive        |
| Cr^{3+}    | 0–30              | 20.71        | 10.30   | Noncompetitive        |
| CTAB       | 0–2.5             | 1.98         | 1.160   | Competitive           |

Figure 4. Lineweaver-Burk graphs substrate pattern of peroxidase from cress (Lepidium sativum sub sp. sativum). (A) Guaiacol substrate, (B) H_{2}O_{2} substrate.

Inhibition properties of the enzyme

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cPOD. The half maximal inhibition concentrations (IC$_{50}$) of these metal ions were found to be 8.36, 20.71, and 31.56 mM, respectively. On the other hand, the inhibition constants of the indicated metals were calculated as 5.78, 10.30, and 20.90 mM, respectively, from Lineweaver–Burk graphs. The inhibition types were determined from these graphs. The inhabitation results were clearly demonstrated that the most effective inhibitor for cPOD was found to be CTAB.

**Conclusion**

In the present study, cPOD was purified from cress (*Lepidium sativum*) using three steps: ammonium sulphate precipitation, CM-Sephadex ion exchange, and gel filtration chromatography. Optimal conditions for the best activity of cPOD were analysed and determined. It was found that cPOD had relatively high activity under acidic conditions but the cPOD is very durable especially under the neutral conditions and the buffer salt concentration slightly affects the activity. Km and Vmax were calculated for both substrates. Also, the effects of different inhibitors on cPOD were determined. It has been demonstrated that cPOD was inhibited as competitive and uncompetitive inhibitions. The information provided here may be useful for purification of POD from different plant sources.

**ORCID**

Ilhami Gülçin [http://orcid.org/0000-0001-5993-1668](http://orcid.org/0000-0001-5993-1668)

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