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Purification and Characterization of Peroxidase from *Brassica oleracea var. Acephala*

İLHAMI GÜLÇİN* and ALI YILDIRIM†

Department of Chemistry, Faculty of Science and Arts, Atatürk University
TR-25240, Erzurum, Turkey
Tel: (90)(442)2314444; Fax: (90)(442)2360948; E-mail: igulcin@atauni.edu.tr

Peroxidase enzyme was extracted and purified from *Brassica oleracea* var. acephala through ammonium sulfate precipitation, dialysis and CM-Sephadex ion-exchange chromatography. The molecular weight of this enzyme was found to be 95 kDa by an SDS-PAGE electrophoresis. Optimum temperature, optimum pH and stable pH of this enzyme were found as 40°C, pH 7.5 and pH 6.5, respectively. The enzyme had K_M values of 5.5 and 1 mM for guaiacol and H_2O_2, respectively. K_M values for pyrogallol and H_2O_2 was found to be 1.92 and 1.25 mM. In contrast K_M values, each of guaiacol/H_2O_2 and pyrogallol/H_2O_2 pairs were 5000 and 833.33 EU/mL, respectively.

Key Words: *Brassica oleracea*, Peroxidase, Purification, Enzyme.

INTRODUCTION

Peroxidase (POD) is a heme protein, which is a member of oxidoreductases [E.C. 1.11.1.7] and catalyzses the oxidation of a wide variety of organic and inorganic substrates in the presence of hydrogen peroxide[1-3]. This enzyme is one of the key enzymes controlling plant growth and development and takes place in various cellular processes including construction, rigidification and eventual lignification of cell walls, protection of tissue from damage and infection by pathogenic microorganisms[4-6]. In the plant kingdom, they serve many purposes, including suberization and wound healing, protection against pathogen attack and the scavenging of damaging hydrogen peroxide from the cell[7, 8]. POD and catalase are two major systems for the enzymatic removal of H_2O_2 and peroxidative damage of cell walls is controlled by the potency of antioxidative peroxidase enzyme system[9, 10]. The peroxidases are capable of oxidizing a broad variety of organic compounds including aromatic amines, indoles and sulfonates using hydrogen peroxide as the oxidant[8, 11-13]. POD is widely used as an important reagent for clinical diagnosis and microanalytical immunoassay because of its

†Department of Chemistry Education, Atatürk University, Kazim Karabekir Education Faculty, 25240 Erzurum, Turkey.

high sensitivity. New applications for POD have been suggested in the medicinal, chemical and food industries[14]. It has been reported that peroxidase has been used for stereospecific biotransformation of a variety of organic molecules[13, 15] and bioremediation[16–20].

Although peroxidases are widely distributed in the plant kingdom, the major source of commercially available peroxidase is roots of horseradish. On the other hand, availability of peroxidases with different specificity would promote the development of new analytical methods and a potential industrial process. In Turkey, *Brassica oleracea var. acephala* (collards) is cultivated mainly in the East Black Sea coast. In the present study, peroxidase enzyme was isolated from this plant, partially characterized and some properties of the enzyme were investigated.

EXPERIMENTAL

Fresh collards (*Brassica oleracea var. acephala*) were harvested in a garden, Giresun, Turkey. Then it was washed, drained, packed in polyethylene bags and stored at –83°C until the enzyme extraction.

Preparation of the homogenate

Unless otherwise noted, all procedures were carried out at 4°C. 10 g leaves of *Brassica oleracea var. acephala* (collards) were removed from frozen storage (–83°C) and ground in a mortar in the presence of liquid nitrogen. This powder was then mixed with 25 mL 0.3 M phosphate buffer (pH 7) and subsequently the collard slurry was centrifuged at 20000 g for 20 min at 4°C. The precipitate was discarded and the obtained supernatant kept at 4°C until use[22].

Ammonium sulphate precipitation and dialysis

Between 0–10, 10–20, 20–30, 30–40, 40–50, 50–60, 60–70 and 70–80% ammonium sulphate precipitation was carried out in a homogenate on an ice bath. Ammonium sulphate was slowly added to the homogenate stirring for completely dissolution. This mixture was centrifuged at 20000 g for 20 min and the precipitate was dissolved in about 3 mL 0.3 M phosphate buffer (pH 7). The concentrated sample was dialyzed for 12 h against 1 L above buffer but contains also 1×10^{-3} M EDTA at 4°C with two changes of dialysis buffer.

Preparation of ion exchange chromatography material

3.5 g dried CM-Sephadex A-50 (Sigma) was dissolved in 100 mL distilled water and incubated in a 90°C water bath for 5 h. Following cooling to the room temperature, this slurry was mixed with 100 mL 0.5 M NaOH and was allowed to stand for 1 h. Afterwards, the supernatant was decanted and the exchanger was washed with distilled water until the effluent was at neutral pH. Then, the exchanger was stirred in 100 mL 0.5 M HCl and allowed to stand for an additional 1 h. Subsequently, the exchanger was washed with distilled water until the effluent was at pH 7.0. Finally, the exchanger was suspended in 0.01 M phosphate buffer (pH 6.5), then packed in a column (3 × 30 cm) and washed and equilibrated with

the same buffer. The flow rates for washing and equilibration were adjusted by peristaltic pump as 40 mL/h[22].

Purification of peroxidase by ion exchange chromatography

The dialyzed and filtered sample was loaded on CM-Sephadex A-50 column previously equilibrated column and the gel was washed with 1 L 10 mM phosphate buffer (pH 6.5). Bound proteins were eluted with a gradient of (250 mL) 0–1 M NaCl in 10 mM phosphate buffer (pH 6.5) at a flow rate of 40 mL/h. Eluates were collected as 5 mL fractions and each of their activity and absorbance were separately measured at 420 nm and 280 nm respectively[23]. Active fractions were pooled and kept at +4°C until use.

SDS-PAGE electrophoresis

SDS-PAGE gel electrophoresis was carried out according to a previously reported method[24] in which there was 3 and 8% acrylamide for stacking and running gel respectively, containing 0.1% SDS.

Peroxidase activity determination

Peroxidase activity was determined by a colorimetric method. Briefly, an aliquot of sample (10–100 µL) was added to a mixture of 1 mL 22.5 mM H_2O_2, 1 mL 45 mM guaiacol and final volume of this mixture was adjusted to 3 mL by addition of 0.3 M phosphate buffer (pH 7). The change in the absorbance at 470 nm was monitored for 3 min at 20°C. One unit of peroxidase activity was defined as 0.01 ΔA_{470} per min[23, 25].

Protein determination

The amount of protein was determined based on dye-binding method of Bradford using bovine serum albumin as a standard[26].

Kinetic studies

The stable pH, optimum pH and optimum temperature were investigated for the enzyme. In order to determine the stable pH and optimum pH, peroxidase enzyme activity was measured in three different buffers: 0.2 M phosphate buffer (pH 4.5–7.5), 0.2 M *tris*-HCl buffer (pH 7.5–9.0) and 0.2 M *tris*-Maleat (pH 7.5–9.0). To be able to find the stable pH, enzyme activity was followed in each of the above buffers at 0.5 pH intervals of indicated pH ranges for 10 days. The optimum temperature was assayed in 0.2 m *tris*-HCl buffer between 0–65°C. K_M and V_{max} values were determined for each of H_2O_2/pyrogallol and H_2O_2/guaiacol substrate pairs. For this, the enzyme activity was measured at five different concentrations of pyrogallol or guaiacol (0.75–15 mM) while H_2O_2 concentration was constant (7.5 mM). In addition, this measurement was performed at a constant concentration of pyrogallol or guaiacol (15 mM) whilst five different H_2O_2 concentrations (3.75–7.5 mM) were used. K_M and V_{max} values were calculated from the plot of 1/V *vs.* 1/[S] by the method of Lineweaver and Burk[27]

RESULTS AND DISCUSSION

Peroxidase enzyme was purified from *Brassica oleracea var. acephala* (collards) with ion exahange chromatography on CM-Sephadex. By applying this method, 33-fold purification was obtained (Table-1). The purity of the enzyme was controlled by SDS-PAGE electrophoresis. There was a single band on this gel and this shows that the peroxidase enzyme was purified (Fig. 1). The molecular weight of this enzyme was determined also by using this electrophoresis band. For this, R_f values of Mw known standard proteins were plotted *vs.* log M_w of these proteins (Fig. 2). Then, R_f value of purified peroxidase was inserted into the equation obtained from the above graph. Thus, the molecular weight of purified peroxidase was calculated as 95000 dalton.

TABLE-1
PURIFICATION STEPS OF PEROXIDASE FROM *BRASSICA OLERACEA VAR. ACEPHALA* (COLLARDS)

Purification steps	Total volume (mL)	Activity (EU/mL)	Total activity (EU)	Total protein (mg)	Specific activity (EU/mg)	Yield (%)	Purification fold
Homogenate	40	620	24800	4.74	1308	100	—
Ammonium sulphate saturation	6	3922	23529	13.59	2885	95	2.20
Dialyze	7	3332	23324	14.56	2888	94	2.20
CM-Sephadex	41.5	198	8217	0.045	43805	33	33.5

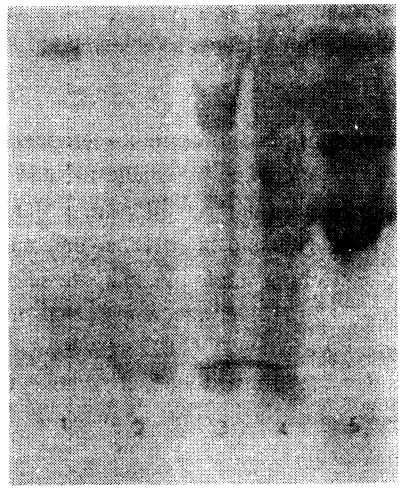

Fig. 1. SDS-polyacrylamide gel electrophoresis of peroxidase from *Brassica oleracea*, where lane 1 is purified peroxidase of collard and lane 5 is standard proteins (*E. coli* β-galactosidase (116000), rabbit phosphorylase B (97400), bovine albumin (66000) and chicken ovalbumin (45000)

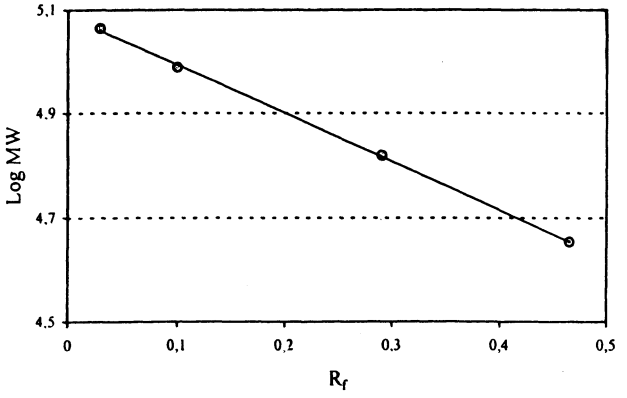

Fig. 2. The MW *vs.* R_f values of standard proteins obtained from SDS-Page electrophoresis bands (*E. coli* β-galactosidase (116000), rabbit phosphorylase B (97400), bovine albumin (66000), and chicken ovalbumin (45000)).

The purification of peroxidase was carried out in a few steps. For this, firstly, several precipitations with solid $(NH_4)_2SO_4$ between 0–10, 10–20, 20–30, 30–40, 40–50, 50–60, 60–70 and 70–80% were carried out to find the proper saturation point. The highest enzyme activity was found in the supernatant of 20–70% ammonium sulphate precipitation. Therefore, this saturation range was used in all of the extraction processes.

Following ammonium sulphate precipitation, the enzyme-containing precipitate was dissolved in 3–5 mL of 0.3 M phosphate buffer (pH 7) and then dialyzed against 1 L, 0.3 M phosphate buffer (pH 7) containing 10^{-3} M EDTA for 12 h. The dialyzed enzyme extract was applied to a CM-Sephadex A 50 ion exchange chromatography and bound proteins were eluted with a linear gradient of 0–1 M NaCl in 10 mM phosphate buffer (pH 6.5) at a flow rate of 40 mL/h. Eluates were collected as 5 mL fractions and each of their activity and absorbance were separately measured at 420 nm and 280 nm respectively (Fig. 3). Active fractions were pooled and kept at +4°C until use.

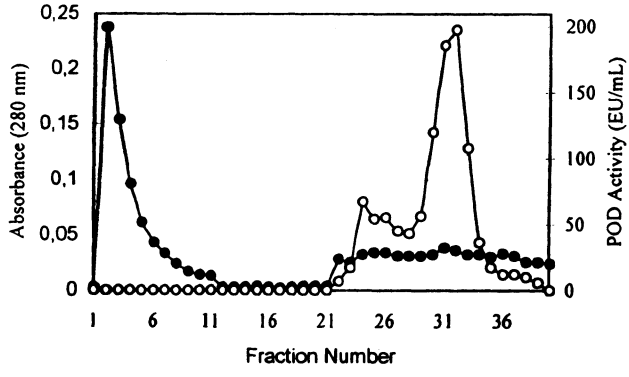

Fig. 3. Ion-exchange chromatography of POD from *Brassica oleracea* on CM-Sephadex A-50 column.

To find optimum pH, enzyme activity was measured in three different buffers: 0.2 M phosphates buffer (pH 5–7.5), 0.2 M *tris*-HCl buffer (pH 7.5–9.0), and 0.2 M *tris*-Maleat buffer (pH 7.5–9.0). It was found that the enzyme activity was highest in 0.2 M *tris*-HCl buffer (pH 7.5) (Fig. 4).

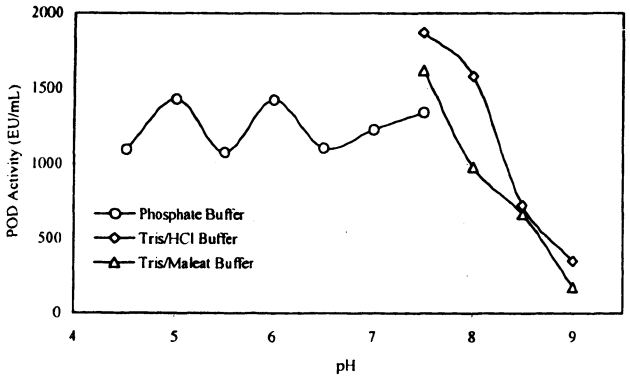

Fig. 4. The effect of pH on the peroxidase activity. The dialyzed enzyme extract was dissolved in indicated buffer and the absorbance change of test sample in 420 nm was measured for 3 min at 20°C.

To be able determine the stable pH of peroxidase enzyme, its activity was followed in three different buffers, having a pH range of pH 4.5–9 at 0.5 pH intervals, for 10 days. As can be seen in Figs. 5, 6 and 7, the enzyme activity was highest in 0.2 M phosphate buffer (pH 6.5), at the end of this incubation period. These results could suggest that although this enzyme has an optimum of pH 7.5, it is the most stable at pH 6.5.

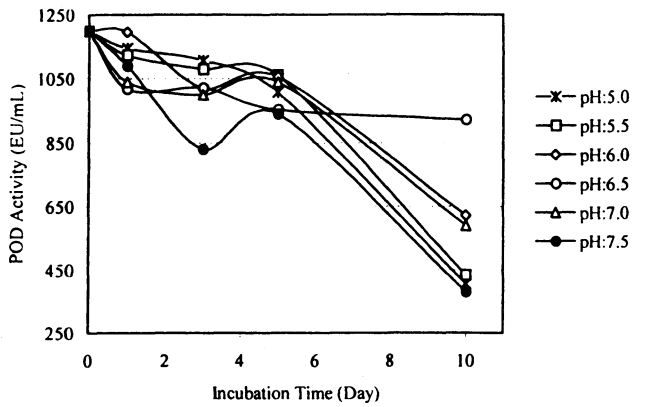

Fig. 5. Effect of incubation period on the activity of peroxidase. Dialyzed enzyme extracts were dissolved in 0.2 M phosphate buffers that indicated the pH and were incubated at +4°C. The peroxidase activity of each of the incubated enzyme solutions was measured at indicated periods.

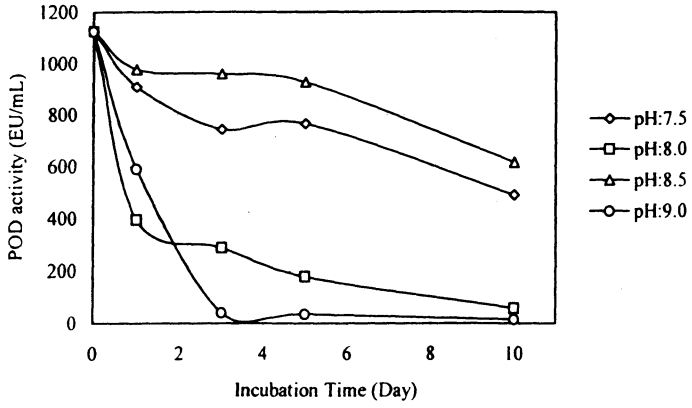

Fig. 6. Effect of incubation period on the activity of peroxidase from *Brassica oleracea*. Dialyzed enzyme extracts were dissolved in 0.2 M *tris*-HCl buffers that indicated the pH and were incubated at +4°C. The peroxidase activity of each of the incubated enzyme solutions was measured at indicated periods.

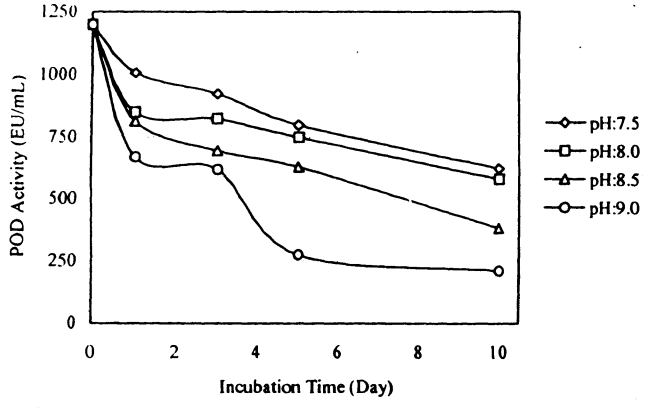

Fig. 7. Effect of incubation period on the activity of peroxidase from *Brassica oleracea*. Dialyzed enzyme extracts were dissolved in 0.2 M *tris*-Maleat buffers that indicated the pH and were incubated at +4°C. The peroxidase activity of each of the incubated enzyme solutions was measured at indicated periods.

By measuring peroxidase activities of enzyme solution (in 0.2 M *tris*-HCl buffer, pH 7.5) that were incubated at corresponding temperatures between 0–65°C for 5 min, the optimum temperature of peroxidase enzyme was determined to be 40°C (Fig. 8). Although this enzyme shows high activity at 45°C as well, above this temperature enzyme activity was decreasing with increasing temperature. The enzyme activity was too low at 65°C, hence above this temperature it was not studied.

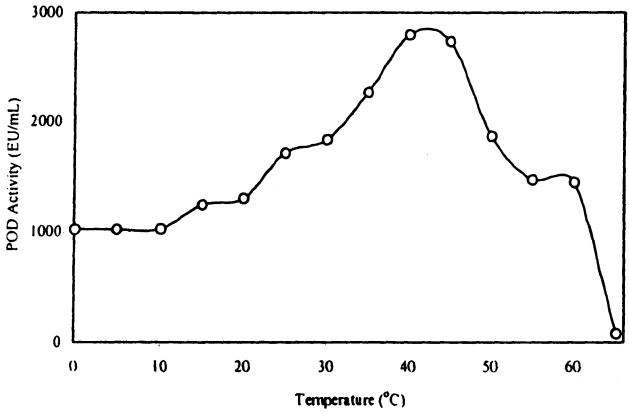

Fig. 8. The effect of temperature on the peroxidase activity. The enzyme activity was determined by measuring absorbance change for 3 min in 0.2 M *tris*-HCl buffer (pH 7.5).

To be able to compare substrate specificity, K_M and V_{max} values were determined for each of H_2O_2/guaiacol and also for each of H_2O_2 and pyrogallol in H_2O_2-pyrogallol substrate pair. For this, the enzyme activities were measured at five different concentrations of pyrogallol or guaiacol (0.75–15 mM) while H_2O_2 concentration was constant (7.5 mM). These measurements were also performed at a constant concentration of pyrogallol or guaiacol (15 mM) whilst five different H_2O_2 concentrations (3.75–7.5 mM) were used, K_M and V_{max} values were calculated from Lineweaver-Burk graphs of the results obtained from above experiments. The enzyme had K_M values of 5.5 mM and 1 mM for guaiacol and H_2O_2, respectively. K_M values for pyrogallol/H_2O_2 were 1.92 mM/1.25 mM. It was of interest to find that V_{max} values for both guaiacol and H_2O_2 were the same in guaiacol/H_2O_2, 5000 EU/mL. The same phenomena occurred in pyrogallol/H_2O_2 pairs and V_{max} values were 833.33 EU/mL for both pyrogallol and H_2O_2. Also it was aimed to find K_m and W_{max} values for each of H_2O_2/ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt) pair. However, there was too low activity of peroxidase in this test system. Therefore, we were unable to determine it. These results suggest that among ABTS, pyrogallol and guaiacol, the most suitable compound, as H-donor, is guiaiacol for the peroxidase enzyme of collard.

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