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Purification and Properties of Peroxidase from *Prangos ferulacea* (Apiaceae) and Investigation of Inhibition by Some Chemicals

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A peroxidase (POD)-containing fraction was purified from *Prangos ferulacea* (apiaceae) by $(NH_4)_2SO_4$ precipitation and anion exchange chromatography. The purification grade obtained was 126.36 yielding 41.8 % of the enzyme activity. The molecular weight was estimated to be 40 kDa by gel filtration. It has an optimum pH of 5 and exhibited high pH and thermal stabilities. Km for guaiacol and ABST were 1.347 and 1 mM, respectively. The optimal temperature was 30 ºC. The I50 values for KSCN and NaN_3 were 0.750 mM, 1.321 mM in *Prangos ferulacea* (Apiaceae) CA, respectively.

Key Words: *Prangos ferulacea* (Apiaceae), Enzyme purification, Characterization, Peroxidase.

INTRODUCTION

Plant peroxidase (EC 1.11.1.7) (POD) is widely distributed in higher plants[1]. These enzymes are involved in a variety of functions, such as control of cell elongation[2], defense mechanisms[3,4] and lignification[5]. On the other hand, POD also plays important roles in food quality, including deterioration of colour and flavour[6]. Peroxidases (EC 1.11.1.7) have been assigned many physiological roles in the several primary and secondary metabolic processes like scavenging of peroxide, participation in lignifications, oxidation of toxic compounds, hormonal signaling, plant defense, IAA catabolism, ethylene biosynthesis and so on[7].

Plant peroxidase has been isolated and characterized from a large number of sources like fruits, leaves, tubers, *etc*. Some of the leaf sources from which POD was purified were barley[8], rice[9], cotton[10], broccoli[11], aloe barbadensis[12], *etc*. Plant peroxidase is widely employed in microanalysis[13,14]. In all cases, multiple isoenzymes have been reported. Isoenzymes purified from these various plant sources differ with respect to molecular mass, thermal stability, pH optimum, substrate specificity and physiological role.

Prangos ferulacea (apiaceae) is among the plants with the highest POD activity, compared to other rich sources such as horseradish[15] and it is an economically important plants for the food industry.

Herein, we report that *Prangos ferulacea* (apiaceae) is one of the richest sources of peroxidase hitherto not studied. In this paper, the characteristics of POD such as substrate specificity, thermal stability and organ distribution, which are important for clarifying the roles of POD, inhibition study of *Prangos ferulacea* (Apiaceae) POD performed by assaying the effect of NaN_3 and KSCN, were determined. In this study, we purified and characterized POD in *Prangos ferulacea* (Apiaceae).

EXPERIMENTAL

Procedure of obtaining plant: *Prangos ferulacea* (Apiaceae) was collected from the land of Erzurum/Oltu, Turkey in May. Then, it was kept in deep freeze until was used.

Extraction of POD: *Prangos ferulacea* (Apiaceae) leaflets were cut into small pieces after removing midribs and milled using a mortar after the removal of panicles. *Prangos ferulacea* (Apiaceae) leaflets flour (25 g fresh weight) was homogenized with 500 mL of extraction buffer containing 50 mM acetate-NaOH buffer (pH 5.0), 1 mM EDTA and 1 mM fresh PMSF for 1 h. A crude enzyme solution was obtained by centrifugation and then precipitated with 60-95 % saturation of solid $(NH_4)_2SO_4$. To increase the purification-fold, the solution was divided into 8 portions before all the steps below were carried out. The precipitate was dissolved in buffer A, which contained 50 mM acetate-NaOH buffer (pH 5.0) and dialyzed overnight against buffer A. The dialyzed enzyme solution was applied to a CM-Sepharose column (2.4 × 11.5 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. The POD was eluted using a linear 600 mL gradient of 50-400 mM NaCl in buffer A. Active fractions were collected and loaded onto a Sephacryl S-200 column (2.4 × 66 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. Active fractions were collected and stored at 30 ºC.

In each purification step, POD activity was measured using guaiacol as a substrate. All of the above steps were carried out at 4 ºC.

Assay of POD: POD activity was determined using an assay system consisting of 20 mM guaiacol (0.5 mL), 0.1 M acetate buffer, pH 5.0 (2.1 mL), 40 mM H_2O_2 (0.2 mL) and the enzyme extract (0.2 mL) with a final volume of 3 mL[4]. Oxidation of guaiacol was measured by the increase in absorbance at 470 nm. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.1/min.

Effect of pH: The enzyme activity pH was determined at different pHs, of 2.0 (200 mM glycine-HCl buffer), 3.0-4.0 (200 mM citrate-NaOH buffer), 5.0-6.0 (200 mM acetate-NaOH buffer), 7.0 (200 mM phosphate-NaOH buffer), 8.0 (200 mM *Tris*-HCl buffer) or 9.0-10.0 (200 mM Borate-NaOH buffer) at 22 ºC using guaicol as a substrate.

Effect of temperature: POD activity was measured at 0-80 ºC using guaicol as a substrate. Reaction mixtures were pre-incubated at each temperature for 0.5 h prior to measuring the POD activity. The enzyme solution was incubated at 0-80 ºC

for 4 h prior to assay and then POD activity was assayed at 22 ºC using guaicol as a substrate. The Km values were determined by Lineweaver-Burk plots at different concentrations of substrate ranging from 0.01-6 mM (guaiacol)[15].

Effect of substrate concentration: Activity of POD at varying concentrations of guaiacol, pyrogallol, ABTS and H_2O_2 were determined and Km values were calculated from Lineweaver-Burk plot.

Thermal stability: Thermal stability experiments were performed by subjecting the enzyme extract to heating at 30-100 ºC. The enzyme extract (2 mL) was taken in separate test tubes and kept at respective temperatures for 1 h. From each tube, an aliquot of 0.2 mL was withdrawn at 5 min intervals and cooled by immersing in ice and was assayed immediately for residual POD activity.

pH Stability: Two mL of enzyme extract in different tubes were adjusted to pH 2-10 by adding1 M HCl or 1 M NaOH. Each sample was kept for 0.5 h at ambient temperature (30-32 ºC). At the end of the experimental period, the pH was adjusted back to initial pH and the residual enzyme activity was assayed as before.

Effect of various compounds on the peroxidative activity of POD: To investigate the effect of various compounds on the peroxidative activity of POD, 1 mM KSCN and NaN_3 compounds were added to the assay mixture, which contained guaicol as a substrate, 200 mM buffer (buffer in optimum pH of POD activity for each substrate), 40 mM H_2O_2 and enzyme solution (about 0.5 ng protein) in a total volume of 3 mL. As a control, reaction mixture without KSCN and NaN_3 was used.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE): Electrophoresis under denaturing conditions was performed at different polyacrylamide gel concentration of 10 % according to the discontinuous buffer system of Laemmli[16]. Electrophoresis was carried out in vertical slab gels and the runs were performed at a constant current intensity of 15 mA/plate in the stacking an d 30mA/plate in the running gels. The molecular weight markers used were bovine serum albumin (66 kDa), albumin egg (45 kDa), pepsin (Porcine stomach mucosa) (34.7 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). The gel was stabilized in the solution containing 50 % propanol + 10 % TCA + 40 % distilled water for 0.5 h. The staining was performed for about 2 h in a solution of 0.1 % Coomassie Brilliant Blue R-250 + 50 % methanol + 10 % acetic acid. Finally, the washing was carried out in a solution of 50 % methanol + 10 % acetic acid + 40 % distilled water until the protein bands were cleared[16].

Estimation of molecular weight of POD: Gel filtration was carried out using the purified POD isozyme. Purified POD was loaded onto a Sephacryl S-200 column (2.4 × 66 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. The molecular weight was determined using a standard curve of elution volume *vs.* log MW derived from standard proteins (bovine serum albumin (66 kDa), albumin egg (45 kDa), pepsin (Porcine stomach mucosa) (34.7 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa).

Protein determination: Total soluble protein concentrations were measured by the method of Bradford[17] using bovine serum albumin is a standard.

RESULTS AND DISCUSSION

Prangos ferulacea (apiaceae) POD was purified 126-fold to homogeneity (confirmed by SDS-PAGE) with a recovery of 42 % (Table-1). POD was found to be precipitated with very high concentrations of $(NH_4)_2SO_4$ in the range of 60-95 %. The 60-95 % $(NH_4)_2SO_4$ precipitate was concentrated, dialyzed against 50 mM acetate-NaOH buffer (pH 5.0) and applied to the CM-Sepharose column. Plant peroxidase bound to the CM-Sepharose and was eluted with a linear gradient of 50-400 mM NaCl in 50 mM acetate-NaOH buffer (pH 5.0) (Fig. 1). This step also facilitated the removal of colour (probably due to phenolics), which was retained in the column yielding a virtually colourless enzyme preparation. Active fractions were collected and stored at 30 ºC.

TABLE-1
PURIFICATION OF PEROXIDASE FROM *Prangos ferulacea* (Apiaceae)

Step	Volume (mL)	Activity (EU/mL)	Total activity		Protein (µg/mL)	Specific activity (EU/mg)	Purification (fold)
			EU	%			
Crude extract	500	40.34	20170	100	0.520	77.58	–
60-95 % $(NH_4)_2SO_4$ precipitate	9	1834.39	16509.51	81.85	0.271	6768.97	87.25
CM-Sepharose column	25	568.64	14216	70.48	0.063	9026.03	116.35
Sephacryl S-200 column	20	421.54	8430.8	41.8	0.043	9803.26	126.36

(2.4 × 66 cm, Amersham Pharmacia Biotech).

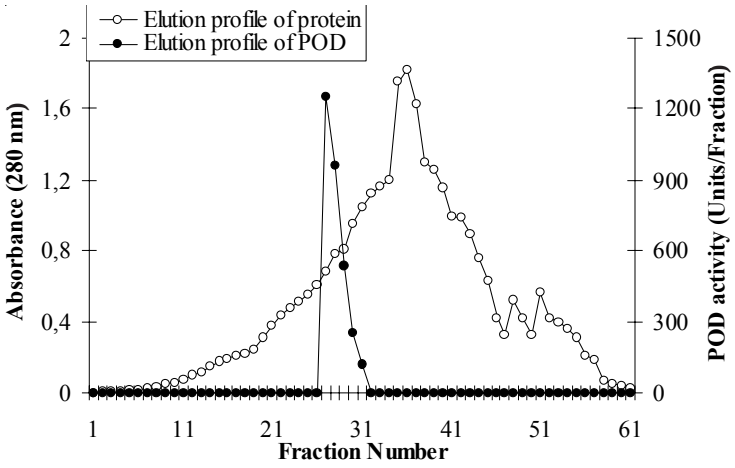

Fig. 1. Ion-exchance chromatography of *Prangos ferulacea* (Apiaceae) on CM-Sepharose column was equilibrated with 5 mM *Tris*-HCl, pH: 8.4 and the 60-95 % $(NH_4)_2SO_4$ precipitate after dialysis against the same buffer was applied to the column and the fractions were eluated at a flow rate of 1 mL/min. Retained proteins were eluted with a linear gradient of 0-0.5 M NaCl in the same buffer. Fractions of 3 mL each were collected and their absorbance at 280 nm and POD activity were checked

The final step of purification was done by Sephacryl S-200 column (2.4 × 66 cm, Amersham Pharmacia Biotech). Active fractions from the above column were concentrated, dialyzed against 50 mM phosphate buffer, pH 6.0 containing 0.5 M NaCl and applied to Sephacryl S 200 column. On eluting the column with the same buffer (Fig. 2), POD came out with the void volume, which indicated a very high molecular weight for the protein probably due to aggregation.

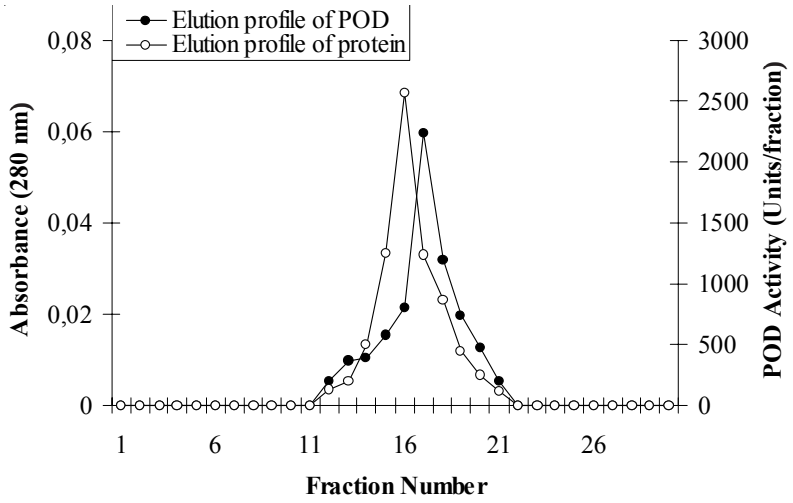

Fig. 2. Molecular exclusion chromatography of *Prangos ferulacea* (Apiaceae) POD on Sephadex G-100: Sephadex G-100 column was equilibrated with 50 mM phosphate buffer, pH: 6.0 containing 0.5 M NaCl. The sample obtained from CM-Sepharose column was dialyzed against the same buffer and applied to the column. Fractions (0.5 mL) at a flow rate of 20 mL/h were collected. The absorbance at 280 nm and POD activity of each fraction were determined.

The above three steps resulted the purification of POD to homogeneity resulting in a fold purification of 126 with a yield of 42 %. The enzyme thus obtained was used for further studies.

By using guaiacol as substrate, POD showed a pH optimum of 5.0 (Fig. 3). An optimum pH of 5 suggests that the enzyme can function in an acidic environment, such as in the vacuole[18]. PODs purified from various sources have their pH optimum mostly in the region of 4.5-6.5. The pH optimum for rice is 5.0 [9], for tomato[19], soybean[20] and coconut PODs[17, 21] it is 5.5 and for strawberry it is 6.0[22].

Plant peroxidase showed greater stability with respect to H^+ ion concentration in the alkaline range. In the pH range 5-10, activity of the enzyme was more or less similar to the original activity. But as the pH was lowered below 5, a sharp decline in stability occurred and at highly acidic conditions the enzyme lost all it's activity. The loss of enzyme activity at low pH was reported to be due to the detachment of haem prosthetic group from the polypeptide chain[23].

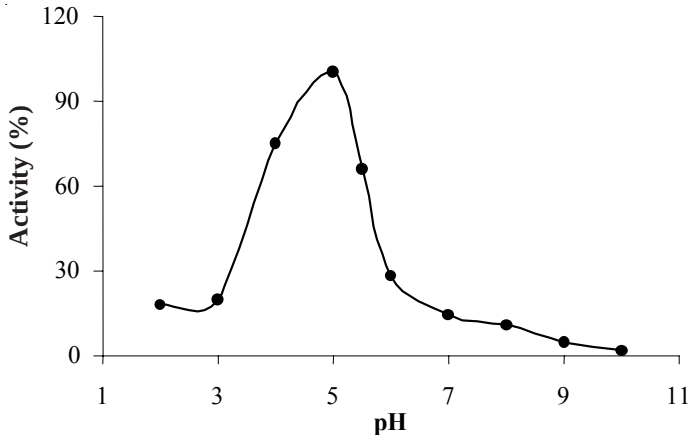

Fig. 3. Effect of pH on activity of *Prangos ferulacea* (Apiaceae) peroxidase.

Prangos ferulacea (Apiaceae) peroxidase exhibited high resistance to heat even at temperature as high as 80 ºC. A major portion of the enzymatic activity was retained at this temperature even after 1 h of incubation. As the temperature was increased from 30 ºC, the resistance exerted by the peroxidase also increased up to 70 ºC after which the temperature declined from 80 ºC onwards (Fig. 4).

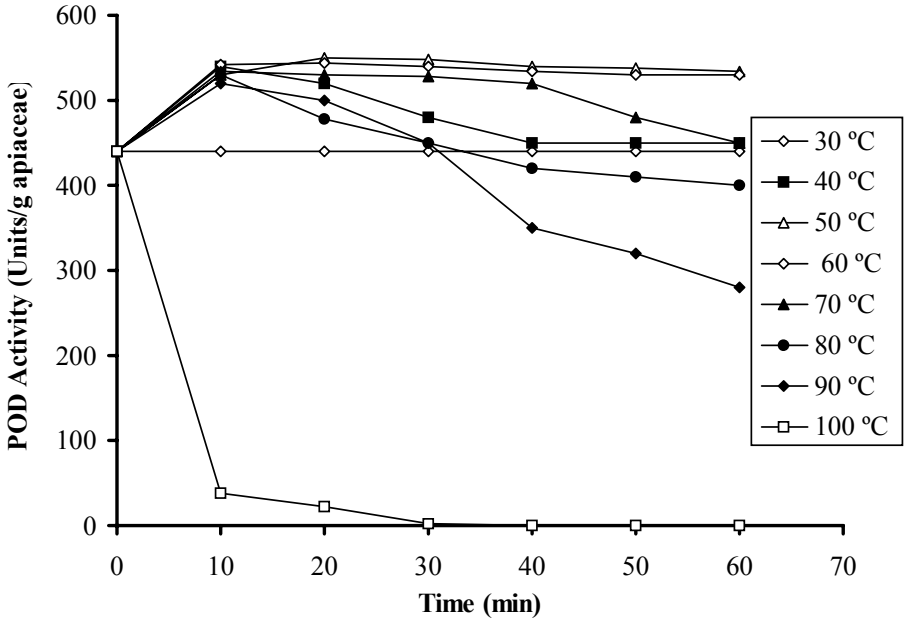

Fig. 4. Thermal stability of purified POD: enzyme extract was kept at respective temperatures (30-100 ºC) in acetate buffer (pH 5.0) for 1 h. Aliquots were taken out at 10 min intervals, immediately cooled in ice and assayed for POD activity. Values plotted on the graph are the mean of three independent experiments. Variation was less than 2 %

Plant peroxidase is reported to be one of the most heat stable enzymes in plants. It was observed that 6 min at 121 ºC is needed to inactivate POD in green peas[15]. However, the resistance to treatment depends on the source of the enzyme as well as the assay conditions, especially pH and nature of substrate employed. Present study shows that the thermal stability of *Prangos ferulacea* (Apiaceae) POD is greater than that reported for cotton[24], strawberry[22] and coconut[21]. It has been shown that the thermal stability of POD is due to the presence of a large number of cysteine residues in the polypeptide chain. It was also suggested that the non-linear inactivation curves are due to the formation of new complexes of higher thermostability formed from thermally denatured enzyme protein and groups of POD that remain active[23]. The thermal stability of cotton leaf peroxidase was reported to be due to the carbohydrate components of the glycoenzyme[24].

Substrate concentration studies carried out using Lineweaver-Burk plot showed an apparent Km of 3.96 mM for guaiacol and 1.3 mM for H_2O_2. Km values for the substrates ABTS and pyrogallol were 1 and 0.84 mM and the Km values for H_2O_2 for ABTS and pyrogallol were 3.8 mM and 5.6 mM, respectively in the present study.

Among the compounds tested, dithiothreitiol at 1 mM concentration inhibited POD activity completely. Sodium azide at 1 mM concentration exhibited only 25 % inhibition. But as its concentration was increased to 20 mM about 98 % inhibition was observed. Sodium thiocyanate also inhibited POD activity (50 %) at lower concentrations (1 mM).

Purified enzyme in 0.1 M acetate buffer, pH 5.0 was scanned in the 250-500 nm range. The spectrum showed two peaks of absorption maxima, one at 403.5 nm (corresponding to ha) and the other at 278.5 nm (for protein). It has an RZ value of 2.3.

Electrophoresis of purified enzyme under denaturing conditions revealed a band for POD corresponding to a Mr of 40 kDa (Fig. 5). The molecular weight of purified POD was also determined by gel filtration on Sephadex G-200 gel filtration column. POD was eluted as a single peak corresponding to an estimated Mr of about 40 kDa.

Present results indicate that *Prangos ferulacea* (apiaceae) POD have molecular weights similar to those reported for horseradish POD (40-46 kDa)[25], broccoli POD (43 kDa)[11] oil palm leaf POD (48 kDa)[26], rice (48 kDa)[9], cotton POD (48 kDa)[24], peanut POD (42 kDa)[23] and tomato POD (43 kDa)[19]. Quite different molecular weights have been reported for PODs purified from, for example, green asparagus (34 kDa)[27] and basic strawberry PODs (58 and 65 kDa)[22]. Molecular weights of PODs from various sources have been reported to range from 30 to 60 kDa and the differences observed are attributed to post-translational modifications of the polypeptide chain including the number and composition of glycan chains present in plant PODs[23,28].

In conclusion, peroxidase has been purified from *Prangos ferulacea* (Apiaceae) and some of its kinetic parameters have been investigated. This study will be useful for future investigation of peroxidase.

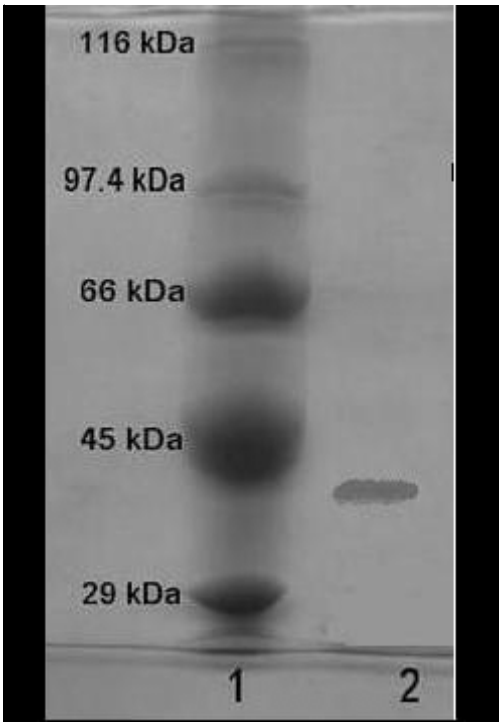

Fig. 5. SDS-PAGE bands of G6PD (Lane 1: standard proteins; 29 kDa, 45 kDa, 66 kDa, 97.4 kDa, 116 kDa, lane 2: prangos ferulacea (apiaceae) POD

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