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# PURIFICATION AND PARTIAL CHARACTERIZATION OF PEROXIDASES FROM CULTIVATED *RAPHANUS SATIVUS L. VAR. CICIL*

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## ABSTRACT

Two peroxidases (EC. 1.11.1.7), POD I and POD II were purified from the roots of cultivated *Raphanus sativus L. Var. Cicil* by one step ion-exchange chromatography after fractionation by acetone. The molecular weight of these enzymes were 43000 and 41000 Daltons and RZ 1.2 and 2.0 for POD I and POD II, respectively. Both enzymes consisted of a single polypeptide chain on SDS-PAGE. The maximum activity of POD I was observed at pH 4.6 and 30°C and for POD II, at pH 6.5 and 60°C. The Km value of POD I for $H_2O_2$ was 7.26 mM and for POD II, 2 mM toward o-dianisidin. Both isoenzymes were stable for 48 hours in temperatures up to 40°C and stable in pH 4-8 for 3 hours.

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**Keywords:** Peroxidase, Horseradish, Purification, Characterization.

## INTRODUCTION

Peroxidase enzymes [donor: $H_2O_2$ oxidoreductase, EC. 1.11.1.7] are widely distributed in plants, animal tissue and micro-organisms.[1-5] Peroxidase was first found in the fig tree in 1936.[6] In 1941 the enzyme was isolated and characterized from horseradish (HRP). During the years of 1942 to 1959, isolation of the enzyme was reported from various sources such as yeast, potato, beans, Japanese radish and wheat.[1]

HRP was first purified by Theorell and his colleagues.[7] Existence of 11 and 7 isoenzymes of horseradish was then reported by Klapper in 1965[1] and Shannon in 1966,[8] respectively. Many researchers have purified peroxidase to apparent homogenicity using extraction, precipitation and fractionation with ammonium sulfate or organic solvent and a combination of anionic and cationic exchange chromatography.[9,10]

The importance of the enzyme is due to its applications as an indicator enzyme for determination of cholesterol,

**Table I.** Comparison of enzyme activity, protein concentration, specific activity and purity number (RZ) of crude extract, acetonic precipitates and purified enzymes.

| | Activity (u/mL) | Protein (mg/mL) | Sp-Act (u/mg) | RZ |
|---|---|---|---|---|
| Crude extract | 258 | 0.397 | 650 | 0.0043 |
| Precipitate 1 vol. acetone | 98 | 1 | 98 | 0.13 |
| Precipitate 2 vol. acetone | 260 | 0.09 | 2888 | 0.404 |
| F1 (POD I) | 57 | 0.010 | 5700 | 1.2 |
| F2 (POD II) | 50 | 0.023 | 2174 | 2.0 |

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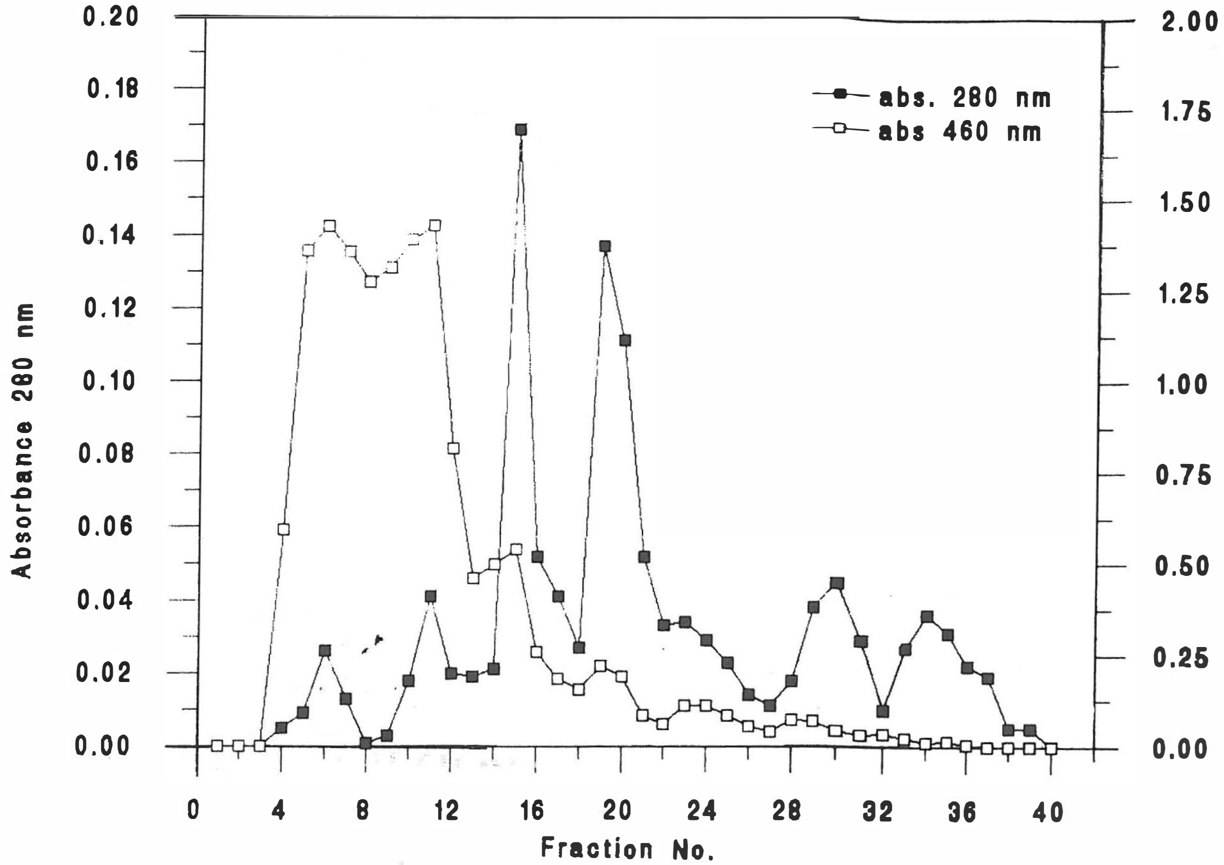


**Fig. 1.** Elution profile of fractions from gel chromatography. The extract was applied to DEAE-Sephadex column (1×30 cm) equilibrated with 10 mM phosphate buffer pH 8.0. Elution was performed by stepwise increasing of NaCl molarity from 0.05 to 0.5 in 10 mM phosphate buffer, pH 8.0. The fraction collector speed was 5 mL/10 minutes.

glucose and many other metabolites in the urine, blood and biological fluids. It the been also used in a conjugated form with antibodies in the ELISA system for measuring hormones in biological fluids.

In our investigation on peroxidase production, due to unavailability of horseradish in Iran, cultivated *Raphanus sativus L. Var. Cicil* was found to be a good source of this enzyme. *R. sativus* is widely cultivated and is available most of the year and its roots are used as a vegetable. This paper presents purification of two isozymes of peroxidase in one step chromatography and some properties of these enzymes.

## MATERIAL AND METHODS

### Extraction of crude enzyme

*R. sativus* roots were purchased from local markets in winter, washed, minced and frozen at -20°C for 24 hrs. The roots juice was extracted by a continuous juice extractor, then passed through cheese cloth and filtered on a 0.45 μm filter. The clear extract was used as a crude enzyme source for purification procedures.

### Enzyme and protein assay

Peroxidase activity was measured spectrophotometrically by the method of Shannon[8] using O-dianisidin as indicator at 460 nm. Protein concentration was determined according to the method of Bradford.[11] During the purification steps, protein concentration was estimated by reading absorbance at 280 nm.

### Enzyme purification

One volume of cold (-20°C) acetone was added slowly to the crude enzyme with continuous stirring. The mixture was allowed to stand for 1 hour at -20°C. The supernatant was collected by centrifugation at 15000 RPM for 10 minutes, and another volume of cold acetone added to it and kept at -20°C. After 24 hours precipitated proteins were collected by centrifugation at 15000 RPM for 10 minutes, dissolved in 10 mM phosphate buffer pH 8.0 and dialyzed against the same buffer at 4°C for 16 hours. The dialyzed protein solutions were loaded on the DEAE-Sepharose (1×30 cm) column which was equilibrated with 10 mM phosphate buffer, pH 8.0. Unabsorbed proteins were eluted by washing the column with 25 mL of starting buffer and absorbed proteins were

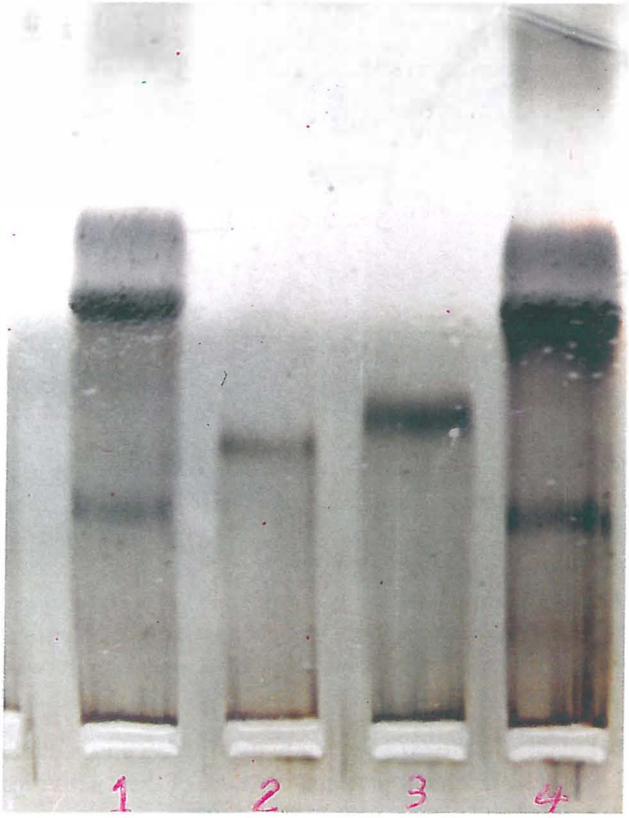


**Fig. 2.** SDS-PAGE analysis of POD I and POD II. 10 μL of concentrated proteins with an equal volume of sample buffer were applied in 10% polyacrylamide gel. Tris-glycine buffer, pH 8.0, was used as running buffer. The electrophoresis was performed with a constant current of 40 mA for 8 hours. Lanes 1 and 4, Pharmacia, LMW size marker; Lane 2, POD I; Lane 3; POD II.

eluted by stepwise increasing gradients of NaCl solution (0.0-0.5 M). In all steps of purifications, RZ (Abs. 460/ Abs. 275) was used as an indication for purity.[12]

**Gel electrophoresis**

10% SDS-PAGE analysis was performed for determination of fractions purity as described by Hames.[13] Concentrated proteins with an equal volume of sample buffer were applied in the gel at a constant current of about 40 mA for 8 hours. The gel was stained with silver nitrate according to the method of Sammons et al.[14]

The molecular weight of proteins was estimated by using Pharmacia low molecular weight size marker.

## RESULTS

Activity of various fractions during acetone fractionation and purification are given in Table I, and the typical elution pattern of peroxidase purification is shown in Fig. 1. Six protein peaks were resolved; F1 to

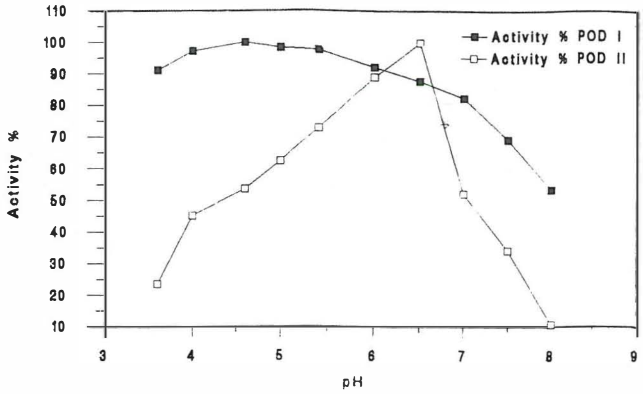


**Fig. 3.** pH-activity profiles of POD I and POD II. The activities of the enzymes were determined at the indicated pHs. 0.05 M acetate buffer is used to maintain pH at 3 to 5, and 0.05 M phosphate buffer to maintain pH at 6 to 8. The test was performed at 30°C for 30 minutes.

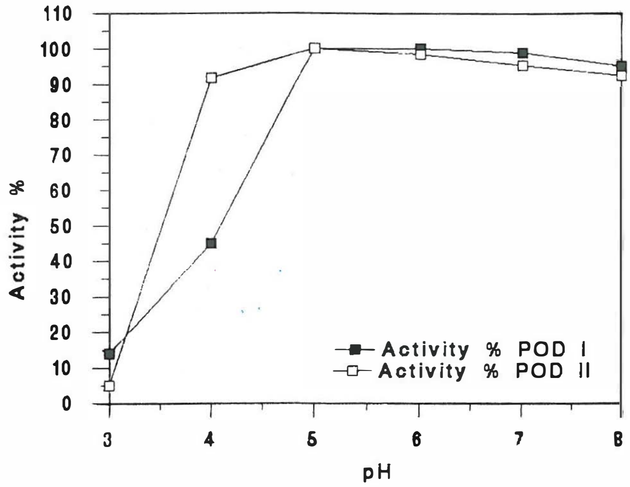


**Fig. 4.** pH-stability profiles of POD I and POD II. The enzymes were incubated in the indicated pHs. 0.1 M citrate buffer was used to maintain pH at 3 to 5, and 0.1 M phosphate buffer was used to maintain pH at 6 to 8. The test was performed at 30°C for 30 minutes.

F6. F1 and F2 had the highest activity, approximately 90% of total activity together, while most of the proteins were in F3 and F4. F1 (tubes 5 to 8) and F2 (tubes 11 to 13) were pooled separately and because of low protein contents, they were concentrated by 2 volumes of cold acetone and dissolved in 1 mL of 10 mM phosphate buffer pH 8.0. SDS-PAGE analysis showed that (Fig. 2) F1 and F2 are pure enzymes with molecular weights of about 43000 and 41000 Daltons, respectively. They were nominated as POD I (F1) and POD II (F2). Their purity was 280 and 470 times more than the crude enzyme with RZ 1.2 and 2.0 for POD I and POD II. Activity of purified enzymes at various pHs (3.6-8.0) was measured and results are shown in Fig. 3. POD I had maximum activity

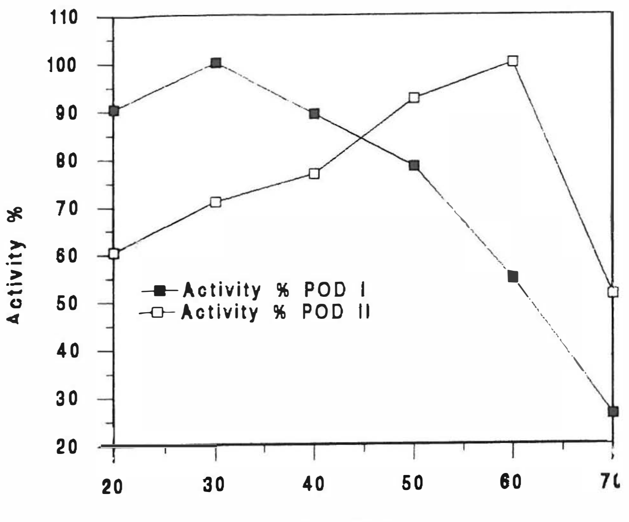


**Fig. 5.** Temperature-activity profiles of POD I and POD II. The activities of the enzyme were determined at the indicated temperatures in 0.05 M acetate buffer, pH 5.4, for 30 minutes.

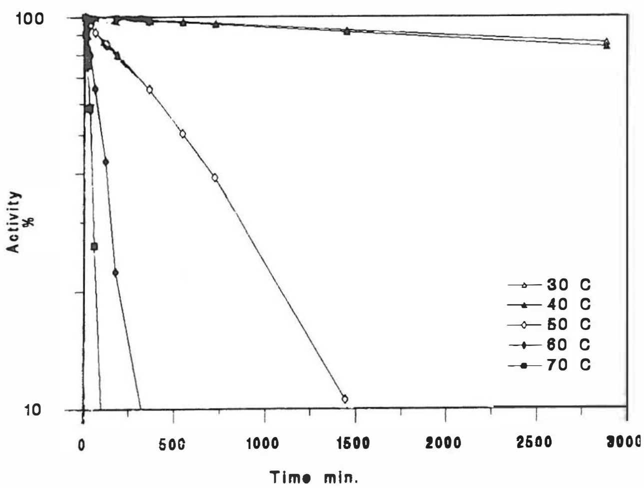


**Fig. 6.** Temperature-stability profile of POD I. The enzyme was incubated in the indicated temperatures in 0.05 M acetate buffer, pH 5.4, for 30 minutes and activity was determined.

at pH 4.6 and POD II at pH 6.5. pH stability of POD I and POD II was determined by incubating the enzymes in 30°C for 180 minutes, at pHs varying from 2.5 to 8.0 (Fig. 4). POD I maintained approximately 90% of its activity at pH above 4.0 and POD II at pH levels above 5.0. As shown in Fig. 4, at pH 4.0, POD I lost more than 85% of its maximum activity whereas POD II was to some extent stable at this pH. Both enzymes nearly lost most of their activities at pH 3.0. Data concerning temperature profile activity in 30 minutes are exhibited in Fig. 5. POD I had a maximum activity at 30°C while POD II showed maximum activity at 60°C.

Results regarding their stability at temperatures

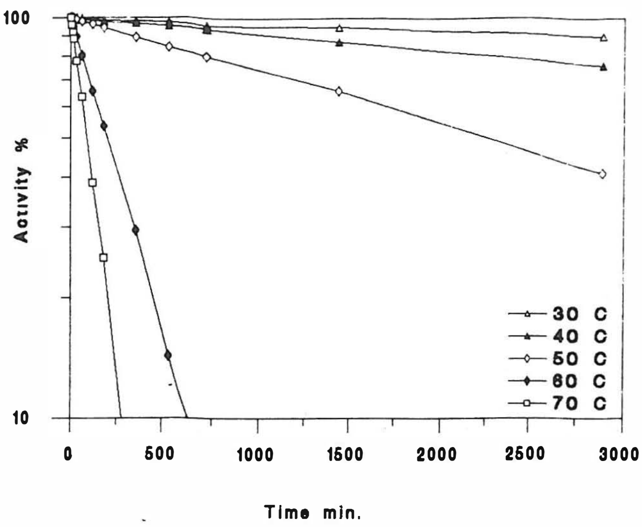


**Fig. 7.** Temperature-stability profile of POD II. The enzyme was incubated in the indicated temperatures in 0.05 M acetate buffer, pH 5.4, for 30 minutes at 30°C, and activity was determined.

between 30°C to 60°C are shown at Figs. 6 and 7. POD I and POD II kept approximately 80% of their activities for 48 hours at temperatures up to 40°C.

## DISCUSSION

The isolated peroxidases from horseradish and many other plants have been extensively studied. Existence of isoenzymes 11 and 7 in horseradish was reported by Klapper and Shannon, respectively.[1,8] Delincee[15] isolated up to 20 isoenzymes from the crude extract of horseradish by thin layer isoelectric focusing and he divided them up to their PI into four groups. Peroxidases isolated from horseradish have molecular weights between 25000-45000 Daltons and a PI of 2.5-9. In the present research we were able to isolate two isoenzymes from the root extract of *R. sativus* and state their purity. The molecular weights of the isoenzymes were 41000 and 43000 Daltons, so they have close similarity with the isoenzymes isolated from horseradish.

Km values of the isoenzymes prepared in this research were 7.26 and 2 mM for POD I and POD II, respectively which were proportionate to those of horseradish peroxidases.[16]

The POD I isoenzyme which was not absorbed by the DEAE-Sepharose column at pH 8 has a positive charge in the mentioned pH, and can probably be categorized under the alkalic isoenzymes while POD II is categorized as a neutral isoenzyme.

By considering that many isoenzymes are usually present in plants, 20 isoenzymes were isolated from horseradish[15] and 12 from pineapple.[17] Our results show that more than 80% of total peroxidase activity has been found in POD I and POD II (Fig. 1), and therefore it can be concluded that these enzymes are the main enzymes and only a few isoenzymes might be in *R. sativus L. Var. Cicil.* The same results were obtained by Prestamo in kiwi fruits and cauliflower peroxidase.[18]

Peroxidase is considered to be a stable enzyme when undergoing thermal treatment; however, resistance to treatment depends on the sources of the enzyme; moreover, in a given source, it varies from one isoenzyme to the other,[19] and also to its degree of glycosylation. Horseradish peroxidase in glycosylated forms has much more thermal stability than non-glycosylated enzyme forms.[20] In our results, it was found that thermal stability for POD I and POD II in temperatures up to 40°C is comparable with the stability of horseradish peroxidase obtained from other sources.[19,21]

## ACKNOWLEDGEMENT

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