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 H₂O₂ 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.

INTRODUCTION

Peroxidase enzymes [donor: H₂O₂ oxidoreductase, EC. 1.11.1.7] are widely distributed in plants, animal tissue and micro-organisms. Peroxidase was first found in the fig tree in 1936. 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.

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

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, acetic precipitates and purified enzymes.

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

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**Fig. 1.** Elution profile of fractions from gel chromatography. The extract was applied to DEAE-Sephadex column (1x30 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.

Enzyme and protein assay

Peroxidase activity was measured spectrophotometrically by the method of Shannon8 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 (1x30 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

**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.
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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 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...
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**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.

**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.

Results regarding their stability at temperatures 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. Delinceel 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.

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 alcalic 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
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.

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, and also to its degree of glycosylation. Horseradish peroxidase in glycosylated forms has much more thermal stability than non-glycosylated enzyme forms. 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.

ACKNOWLEDGEMENT

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REFERENCES
