Properties of Two Anionic Peroxidase Isoenzymes from Turnip (*Brassica napus* L.) Roots

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Two anionic peroxidase isoenzymes of turnip (*Brassica napus* L.) roots (A1 and A2) have been partially purified to study some properties which may be beneficial for their use in clinical and enzyme immunoassays. Both isoenzymes showed similar thermostability in preincubation tests or storage at 0−4 °C and −20 °C, which was comparable to that reported for other peroxidase isoenzymes. The optimum pH for the reaction with ABTS as substrate was 4.3 for both isoenzymes while it was 5.3 for A1 and 4.6 for A2 when *o*-dianisidine is used. Isoenzyme A1 showed higher affinities for the substrates *o*-dianisidine (K_m 0.25 mM) and ABTS ($S_{0.5}$ 50 μM) than A2 (K_m 0.37 mM and $S_{0.5}$ 158 μM, respectively). Nevertheless the affinity of both isoenzymes for ABTS was 80 and 25 times higher, respectively, than that described for commercially available anionic peroxidases.

Keywords: *Brassica napus L.; peroxidase isoenzymes; turnip*

INTRODUCTION

Plant roots are frequently used as sources of pharmaceutical products, agrochemicals, flavors, dyes, and fragances. Peroxidases (EC 1.11.1.7), especially from plant roots, have been widely used in clinical biochemistry and enzyme immunoassays (Krell, 1991). Horseradish *(Armoracia rusticana* L.) root tubers produce the most commonly employed enzyme for commercial purposes (Yamada et al., 1987; Saitou et al., 1991). However other cultivated species may provide peroxidases exhibiting similar or better substrate specificities, thermostability, or yield and economic feasibility (Macek et al., 1993).

Here we describe the partial purification of two anionic peroxidase isoenzymes isolated from turnip (*Brassica napus* L.) roots, their thermostability during storage or preincubation in buffer, and the kinetic properties with *o*-dianisidine and ABTS as substrates.

These properties are important in the characterization of these peroxidase isoenzymes and the evaluation of their potential use for clinical purposes and production of enzyme immunoassays reagents.

MATERIALS AND METHODS

Freshly collected turnip roots were purchased from the local market and immediately processed or frozen at −20 °C until used.

Chemicals. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Crude Extracts. Crude extracts were obtained by grinding the roots at 0−4 °C in a mortar with 2 mL/g of fresh tissue of 10 mM acetate buffer (pH 4) and 25% w/w Polyvinylpyrrolidone (PVP). The extracts were centrifuged at 3500*g* for 5 min, and the supernatants were used as crude extracts for further purification.

Purification of Peroxidase Isoenzymes. Purification of peroxidase isoenzymes was performed by ion-exchange chromatography on DEAE Sephacel columns. Crude extracts were dialyzed overnight, at 0−4 °C, against 10 mM HCl-Tris buffer (pH 8.6), in cellulose membranes which retained proteins of mol wt 12.000 or greater. Samples of 3−4 mL were loaded on a DEAE Sephacel column (1 × 24 cm) previously equilibrated in the same buffer. The column was washed with 45 mL of buffer, and the proteins were eluted with 200 mL of a linear salt gradient (0−0.3 M NaCl) in the same buffer, at 10−15 °C and a flow rate of 1 mL/min controlled with a peristaltic pump (LKB 2232 Microperpex).

Polyacrylamide Gel Electrophoresis. Electrophoretic patterns of the crude extracts and purified fractions were obtained by anionic PAGE on 7.5% polyacrylamide gel slabs at pH 8.3 (Davis, 1964), and the peroxidase activity was detected by staining with benzidine as previously described (De Forchetti and Tigier, 1990).

Peroxidase Activity. Peroxidase activity with *o*-dianisidine as substrate was determined in a 1 mL reaction mixture containing 0.63 mM *o*-dianisidine, 0.5 mM H_2O_2, 100 mM acetate buffer (pH 4.6 or 5.3), and 1−2 μL of crude extract or 5−10 μL of the purified fractions. Peroxidase activity was measured by a continuous spectrophotometric method, following the increase in absorbance at 470 nm produced by oxidation of *o*-dianisidine at 35 °C (ϵ_{470nm}: 11.3 mM^{-1} cm^{-1}). With 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate, the reaction mixture contained 5 mM ABTS, 0.6 mM H_2O_2, 2 μL of the purified fraction, and 100 mM HCl−glycine buffer, pH 4.3. Activity was measured following the continuous increase in A_{414} nm at 30 °C (ϵ_{414nm}: 31.1 mM^{-1} cm^{-1}), produced by the oxidation of ABTS. One unit of enzyme is defined as the amount of enzyme forming 1 μmol of product in 1 min at the temperature and pH specified for each reaction. The spectrophotometric assays were carried out with a Beckman DU-65 thermostatized spectrophotometer.

Protein Determination. Proteins were determined in each column fraction by their A_{280}, and in crude extracts by the method of Bradford (1976) using bovine serum albumin as standard.

Enzyme Thermostability. For the determination of the enzyme thermostability during incubation, 2−5 μL of each fraction were preincubated for 10 min at different temperatures (35−90 °C) in acetate buffer, pH 5.3 for A1 or pH 4.6 for A2. Then, residual activity was determined with *o*-dianisidine as yet described, and it was expressed as percentage of initial enzyme activity.

For the determination of thermal stability during storage, 100 μL aliquots of a partially purified fraction from turnip were kept at 0−4 or −20 °C, and the activities were determined every 4 weeks for 6 months, as described above.

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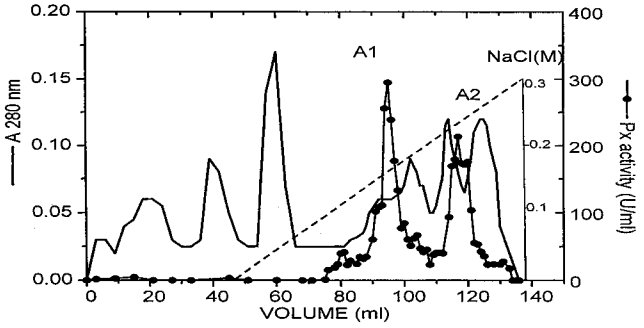

Figure 1. Elution profile of DEAE Sephacel column of crude extracts from turnip roots performed as described in Materials and Methods: (—) proteins (A_{280nm}); (-·-) peroxidase activity with *o*-dianisidine as substrate (unit/mL); salt gradient applied (NaCl, 0−0.3 M).

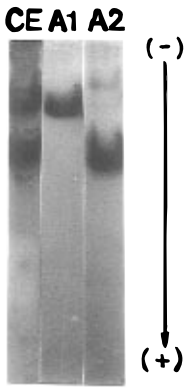

Figure 2. Electrophoretic patterns of anionic PAGE for crude extracts (CE) and fractions A1 and A2 obtained from the DEAE Sephacel column stained with benzidine to detect peroxidase activity.

RESULTS

Purification and Separation of Isoenzymes. The elution profile through a DEAE Sephacel column of crude turnip roots extracts is shown in Figure 1. Two peaks (A1 and A2) of peroxidase activity with *o*-dianisidine as substrate were obtained, corresponding to each of the two main anionic isoenzymes detected by anionic PAGE in crude extracts (Figure 2).

Kinetic Studies. Kinetic studies were performed with both isoenzymes using *o*-dianisidine or ABTS as substrates. Although *o*-dianisidine is a common electron donor used for the measurement of peroxidase activity, it is not recommended as a substrate for enzyme immunoassays since its solubility is low and because it is sensitive to ammonium salts (Tijssen, 1993). So, it was necessary to try another electron donor like ABTS, more appropriate for those assays and commonly employed in commercial kits.

Figure 3A,B shows the results of the kinetic studies performed with *o*-dianisidine (Figure 3A) or ABTS (Figure 3B) as substrates. Insets are Lineweaver−Burk plots (Figure 3A) or Hill plots (Figure 3B) for K_m or $S_{0.5}$ estimation, respectively. Both isoenzymes showed Michaelis−Menten kinetics with *o*-dianisidine as a substrate, while the substrate curves for ABTS were slightly sigmoidal. The kinetic constants and the optimum pH for each isoenzyme in both reactions are shown in Table 1. Isoenzyme A2 had the lowest substrate affinity for *o*-dianisidine and ABTS compared with A1.

Thermostability of Purified Peroxidase Isoenzymes. Peroxidase isoenzymes A1 and A2 retained 50% of their activity after incubation at 55−60 °C for 10 min

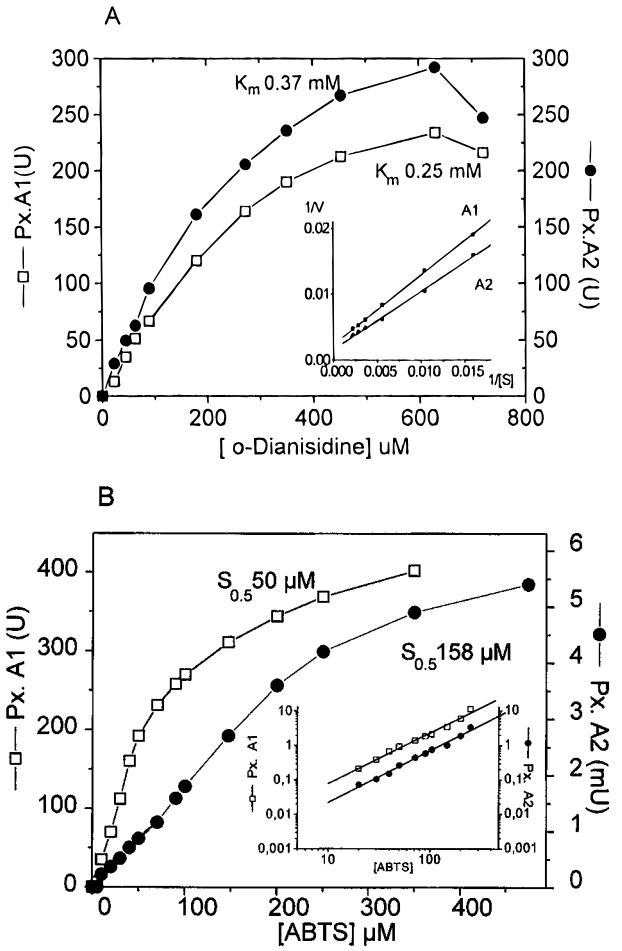

Figure 3. *o*-Dianisidine saturation curve for A1 and A2 isoenzymes (A) and ABTS saturation curve (B). Inset: Lineweaver−Burk and Hill plots for K_m and $S_{0.5}$ estimation.

Table 1. Kinetic Properties of Turnip Anionic Isoenzymes[a]

property analyzed	A1	A2
Substrate: *o*-Dianisidine		
optimum pH	5.3	4.6
K_m for *o*-dianisidine (mM)	0.25	0.37
K_m for H_2O_2 (mM)	1.33	1.40
Substrate: ABTS		
optimum pH	4.3	4.3
$S_{0.5}$ for ABTS (mM)	0.050	0.158
K_m for H_2O_2 (mM)	0.2	0.018

[a] For optimum pH estimation, both 100 mM sodium acetate buffer (in the pH range 3.5−5.5) and 100 mM sodium phosphate buffer (for the pH range 5.7−7.5) were used.

in buffered solution (Figure 4A,B) as reported for commercial peroxidase isoenzymes (Moreno et al., 1990).

During storage at 0−4 °C or −20 °C, both peroxidase isoenzymes retained full activity for at least 6 months.

DISCUSSION

Brassica napus root extracts contain two anionic peroxidase isoenzymes which could be purified and separated by ion-exchange chromatography on a DEAE Sephacel column.

The main anionic isoenzymes (A1 and A2) showed Michaelis−Menten kinetics with *o*-dianisidine as substrate, with different optimum pH and K_m of 0.25 and 0.37 mM, respectively. These affinities were higher than those reported for Korean-radish root anionic

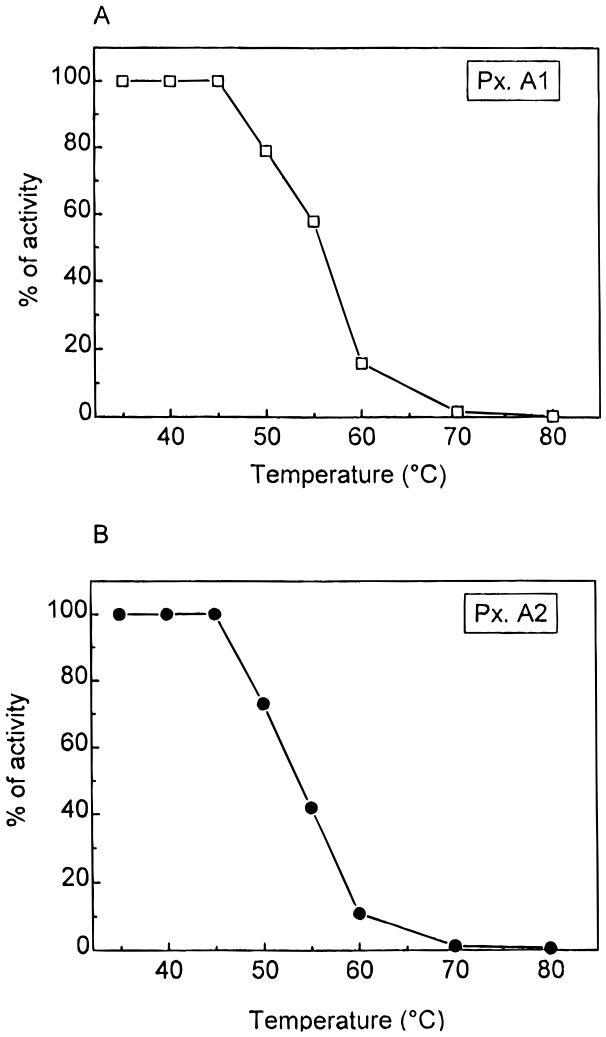

Figure 4. Thermal inactivation of isoperoxidases A1 (A) and A2 (B) during 10 min preincubation at different temperatures.

isoenzymes (Lee et al., 1994) and similar to those of tobacco (Mäder et al., 1986).

Substrate curves were slightly sigmoidal for both isoenzymes when the electron donor was ABTS, although with the same optimum pH. Nevertheless, A1 showed higher affinity than A2 for this substrate, and both isoenzyme affinities were 80 and 25 times higher, respectively, than those reported for HRP A1 and A2 anionic isoenzymes commercially supplied (BZ HRP5, Sigma type VII and Sigma type VIII) (Hiner et al., 1996). These properties would be of interest to differentiate both isoenzymes and to study their potential industrial applications.

The optimum pH for each reaction was similar to that previously reported for other peroxidase isoenzymes (Arnao, 1990; Moreno et al., 1990; Lee et al., 1994). A1 and A2 showed similar thermostabilities during storage or incubation in buffer, which were comparable to that of the commercially available HRP (Moreno et al., 1990).

It is our interest to improve the extraction and purification of these isoenzymes using methods more adequate for industrial production of enzymes and various strategies are under investigation in our laboratory.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase.

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