Journal of Plant Physiology **162** (2005) 609—617

Available online at www.sciencedirect.com

SCIENCE DIRECT®

JOURNAL OF PLANT PHYSIOLOGY

www.elsevier.de/jplph

Purification and characterization of a cationic peroxidase C_S in *Raphanus sativus*

Soung Soo Kim[a], Dong Ju Lee[b,*]

[a]*Department of Biochemistry, College of Science, Yonsei University, Seoul 120-749, Korea*
[b]*MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA*

Received 24 August 2004; accepted 13 October 2004

KEYWORDS
Cationic peroxidase;
o-Dianisidine;
Hydrogen peroxide;
Purification;
Raphanus sativus;
Reconstitution;
Seed-specific

Summary

A short distance migrating cationic peroxidase from Korean radish seeds (*Raphanus sativus*) was detected. Cationic peroxidase C_s was purified to apparent homogeneity and characterized. The molecular mass of the purified cationic peroxidase C_s was estimated to be about 44 kDa on SDS-PAGE. After reconstitution of apoperoxidase C_s with protohemin, the absorption spectra revealed a new peak in the Soret region around 400 nm, which is typical in a classical type III peroxidase family. The optimum pH of peroxidase activity for *o*-dianisidine oxidation was observed at pH 7.0. Kinetic studies revealed that the reconstituted cationic peroxidase C_s has K_m values of 1.18 mM and of 1.27 mM for *o*-dianisidine and H_2O_2, respectively. The cationic peroxidase C_s showed the peroxidase activities for native substrates, such as coumaric acid, ferulic acid, and scopoletin. This result suggested that cationic peroxidase C_s plays an important role in plant cell wall formation during seed germination.

Introduction

The basic reaction catalyzed by plant peroxidases (EC 1.11.1.7, donor: hydrogen peroxide oxidoreductase) utilizes hydrogen peroxide produced in metabolic reactions to oxidize a variety of aromatic compounds (Anderson et al., 1991). Peroxidases are widely distributed in organisms, and plants have an abundance of peroxidase isozymes which have been implicated in a wide variety of cellular functions, including lignification (Quiroga et al., 2000), suberization (Bernards et al., 1999), auxin oxidation (Gazaryan et al., 1996), and plant defense (McLusky et al., 1999). Recently, wheat flour peroxidase has been identified as a new allergen (Sánchez-Monge et al., 1997). Plant peroxidases belonging to classes II and III are single-chain proteins that are often highly

Abbreviations: AP, anionic peroxidase; CP, cationic peroxidase; DAG, day after germination; PMSF, phenylmethylsulfonylfluoride

*Corresponding author. Tel.: +1 517 353 0697; fax: +1 517 353 9168.
E-mail address: leedong@msu.edu (D.J. Lee).

0176-1617/$ - see front matter © 2005 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2004.10.004

glycosylated (Lee and Kim, 1993; Lee et al., 2001; Lige et al. 2001). Most of the peroxidase action occurs at the cell walls, where these enzymes may be involved in modulating cell wall rigidity and extensibility. However, the role that peroxidase plays in metabolism is not clear because of the large number of reactions that it catalyzes and the considerable number of isoenzymic species. Hejgaard et al. (1991) reported that barley peroxidase, BP1, purified from mature barley grain, is a classical plant peroxidase. BP1 was isolated as two variants, a glycosylated form, BP1a ($Mr =$ 37 kDa) and a non-glycosylated form, BP1b ($Mr = 36$ kDa) (Rasmussen et al., 1991). Several reports have shown that horseradish peroxidase was well reconstituted with hemin and regained its peroxidase activity (DiNello and Dolphin, 1981). Baldini et al. (2002) reported that reconstituted horseradish peroxidase with synthetic protohemins was less active than native horseradish peroxidase. In general, it has been observed that the reconstituted proteins exhibit reduced catalytic activity.

In the case of the Korean radish root, two cationic isoperoxidases (termed C_1 and C_3) and four anionic isoperoxidases (designated A_1, A_2, A_{3n}, and A_3) had been purified homogeneously and characterized (Lee and Kim, 1994). The physiological, kinetic, and immunological properties of these isoperoxidases also have been studied (Lee and Kim, 1993, 1994; Kim and Kim, 1996). All six isoperoxidases are glycoproteins composed of a single polypeptide chain. The optimum pH values against guaiacol range between pH5.0 and 6.5. All these isoperoxidases follow the simple Michaelis–Menten kinetics for guaiacol and *o*-dianisidine oxidation. The N-terminal amino acid sequences of all isoperoxidases were sequenced except A_2. In this report, a novel cationic peroxidase (designated C_s) from Korean radish seed was purified, reconstituted, and characterized in terms of its physiological, kinetic, and catalytic properties.

Materials and methods

Plant materials

The mature Korean radish seeds (*Raphanus sativus* L. cv F1 Handsome Fall) were bought from Seoul Seed Co., Ltd., Korea. Seeds were homogenized in 4 times (v/w) of ice-cold 50 mM sodium phosphate buffer (pH 6.0) plus 5 mg L^{-1} phenylmethylsulfonylfluoride (PMSF) using a porcelain pestle and mortar with clean sea sand as an abrasive. After centrifugation at 5000*g* for 10 min at 4 °C to remove cell debris, ammonium sulfate was added little by little to the supernatant to 90% saturation and kept at 4 °C overnight. The protein pellet, collected by centrifugation at 15,000*g* for 30 min at 4 °C, was resuspended in a minimal volume of distilled water and dialyzed against a 10,000 × volume of 5 mM sodium phosphate buffer (pH 6.0). The supernatant was used as the source of peroxidase after removing insoluble materials by centrifugation at 15,000*g* for 30 min at 4 °C.

Raising antibody

To purify cationic peroxidase C_s using an immunoaffinity method, we prepared antiserum against cationic peroxidase C_3 purified from Korean radish root, which was homogeneous on SDS-PAGE. For the primary immunization, rabbits were injected subcutaneously with 0.2 mg mL^{-1} of the purified cationic peroxidase C_3 emulsified with an equal volume of Freund's adjuvant. Subsequent booster immunization with the same antigen emulsified in Freund's incomplete adjuvant was performed at 2 weeks after the first immunization, and the rabbits were sacrificed 2 weeks after the booster immunization.

Enzyme purification

The purified polyclonal anti-peroxidase C_3 antibodies were covalently attached to a solid-phase matrix according to the method from BIO-RAD. All purification steps were carried out at 4 °C. The soluble extract was applied to an immunoaffinity column (3 mL) pre-equilibrated with a washing buffer 1 (10 mM Tris buffer, pH 8.0, 140 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate). After the column was washed with 5 column volumes of washing buffer 1 and washing buffer 2 (50 mM Tris buffer, pH 9.0, 0.5 M NaCl, 0.1% Triton X-100) at a flow rate of 1 mL min^{-1}, the binding protein was eluted with 3 column volumes of elution buffer (50 mM Triethanolamine, pH 11.5, 150 mM NaCl, 0.1% Triton X-100) following monitored at 280 nm. After fractions (0.8 mL) were neutralized with 0.2 mL of 1 M Tris buffer (pH 6.7), the protein fractions were collected, dialyzed against 50 mM sodium phosphate buffer (pH 6.0), and then used. The affinity column was subsequently washed with 5 column volumes of washing buffer.