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Enzymatic Properties of Fast-migrating Cationic Peroxidase Isozyme from Rice Callus

Kyung A Yoo, Mi Young Lee*

Department of Life Science, College of Science, Soonchunhyang University, Asan PO Box 97, Chungnam, 336-600, Korea.

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Abstract

The fast-migrating cationic peroxidase isozyme, named RC3, was purified from rice (*Oryza sativa* cv. Nak-Dong) callus. Purification of the enzyme was accomplished by ammonium sulfate fractionation, CM-cellulose ion-exchange chromatography, and Sephacryl S-100 gel filtration. The molecular mass of the enzyme was about 34 KDa as determined by SDS-PAGE and 38 KDa by Sephacryl S-100 gel filtration. The pI value of the enzyme was 8.9. Antiserum against RC3 was raised in rabbits, and anti RC3 antiserum reacted with RC3 isozyme by Ouchterlony double immunodiffusion. The optimum pHs and Km values of the enzyme for various substrates were determined. Kinetic studies with various substrates showed that RC3 had very low Km value of 0.01 mM for ferulic acid and ascorbic acid. However, the enzyme did not use esculetin as a substrate.

Introduction

Plants contain abundant amounts of multiple peroxidases [POD, EC 1.11.1.7] that exhibit broad substrate specificities (van Huystee and Cairns, 1982). PODs have been implicated in a wide range of cellular reactions such as phenolic compound oxidation, indole-3-acetic acid oxidation, lignification and polysaccharide cross-linking. Obviously, they play a key role in plant growth and development (van Huystee, 1987). Moreover, they are known to be related with the defense mechanisms toward pathogens and various stresses including metal ion (Antje et al., 1996), salt (Bakardjieva et al., 1996; Yi et al., 2002) and air pollution damage (Castillo et al., 1987; Lee, 2002). Studies on the gene structure, overexpression and promoter function of individual POD isozymes have been intensively studied in various plants such as tobacco (Dowd et al., 2000), *Arabidopsis* (Tognolli et al., 2000), scented-geranium (Lee et al., 2001) , Korean radish (Park and Kim, 1996; Lee and Kim, 1998), sweet potato (Huh et al., 1997; Kim et al., 1999) as well as rice (Hiraga et al., 2000; 2001).

According to the classification by Welinder (Dunford, 1999; Hiraga, 2001), classical secretory plant PODs such as horseradish POD (HRP), peanut POD and barley POD belonged to the Class III POD superfamily. They have two conserved calcium ions, an N-terminal signal peptide, four conserved disulfide bridges and 0 to 25% carbohydrate content. However, distinct differences in physicochemical and catalytic properties have been reported among POD isozymes even within one plant family (Lee and Kim, 1994).

In the case of rice (*Oryza sativa* L.), four POD isozymes from green leaves were isolated and characterized (Ito et al., 1991). Moreover, rice cationic POD, PO-C1, which was induced in incompatible interactions between the vascular pathogen *Xanthomonas oryzae pv oryzas* and rice, was also purified (Scott et al., 1995). The present study describes the purification of the fast-migrating POD isozyme, named RC3, from rice callus. Some of its enzymatic and physicochemical properties have been also investigated.

* Corresponding author; E-mail; miyoung@sch.ac.kr
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Materials and Methods

Experimental plant

Rice (*Oryza sativa* cv. Nak-Dong) seeds were sterilized with 50% sodium hypochlorite for 10 min and washed extensively with distilled water. The seeds were placed in N6 media at 27℃ under dark condition. After 15 of days of incubation, uniformly induced callus was selected. The rice callus was aseptically transferred to the same medium and then grown for 14 days.

Peroxidase (POD) assays and protein determination

The POD activity with guaiacol as a substrate was assayed as previously reported (Lee and Kim, 1994). The assay mixture contained 40 mM phosphate buffer, 15 mM guaiacol, 5 mM H_2O_2 and 50 uL of enzyme preparation in a total volume of 1 mL. The reaction was initiated by the addition of H_2O_2, and the increase in absorbance at 470 nm was measured using a UV/VIS spectrophotometer. Typi cal assay conditions and wavelengths for various substrates including o-dianisidine, ferulic acid and esculetin were determined as previouly reported (Lee and Kim, 1994). Protien was determined by the method of Lowry et al. (1951).

Gel electrophoresis

Starch gel electrophoresis was performed as described previously (Lee and Kim, 1994). POD bands were visualized by placing the gel in a solution of 100 mg of 3-amino-9-ethylcarbazole in 10 mL of N,N-dimethyl-formamide, 184 mL of acetate buffer (pH 5.0), 10 mL of 100 mM $CaCl_2$ and 0.2 mL of 30 % H_2O_2. The same quantities of proteins were loaded for all of the gel analysis. SDS-polyacrylamide gel eletrophoresis was carried out as described by Laemmli (1970).

Preparation of crude enzyme

Rice callus homogenate in 5 mM sodium phosphate buffer (pH 6.0) was adjusted to 80% saturation with $(NH_4)_2SO_4$. The pellet from $(NH_4)_2SO_4$ treatment was then dissolved in minimum volume of 30 mM sodium phosphate buffer (pH 6.0) and dialyzed against the same buffer.

Enzyme purification

The crude enzyme was loaded on a CM-cellulose ion exchange column (3.5 × 12 cm) preequilibrated with 30 mM sodium phosphate buffer (pH 6.0). The column was washed with the same buffer until the absorbance of the eluent containing all anionic POD isozymes and cationic POD isozymes named RC1 and RC2 at 280 nm became zero. POD isozyme RC3 was eluted with 50 mM sodium phosphate buffer after complete elution of RC1 and RC2. The fractions containing POD isozyme RC3 were lyophilized and dialyzed against 50 mM sodium phosphate buffer (pH 6.0). The lyophilized sample was applied to a Sephacryl S-100 column (1.3 × 110 cm) preequilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was then eluted with the same buffer in order to obtain purified POD isozyme RC3.

Molecular mass determination

The molecular mass of POD isozyme RC3 was estimated by gel filtration chromatography on Sephacryl S-100 column (1.5 × 78 cm). The subunit molecular mass of the enzyme was determined by SDS-polyacrylamide gel electrophoresis.

Determination of isoelectric point

The isoelectric point (pI) of RC3 was determined as previously reported (Lee and Kim, 1994). A suitable amount of sample was applied on the polyacrylamide gel plate containing ampholine carrier-ampholite (pH 3.5- pH 9.0).

Preparation of anti RC3 antiserum

Purified RC3, which was homogeneous on polyacrylamide gel electrophoresis, was used as the antigen as reported earlier (Lee et al., 1994). For the primary immunization, a rabbit was injected subcutaneously with 500 μg of RC3 emulsified with Freund's complete adjuvant. Subsequent booster immunization was performed at 10 days after the primary immunization, and the rabbit was sacrificed 10 days after the booster immunization. Immune antiserum was collected by centrifugation and used for this experiment.

Ouchterlony double immunodiffusion

Ouchterlony double immunodiffusion was performed at room temperature in a 1% agarose gel. Proper amounts of POD isozyme RC3 were incubated with anti RC3 antiserum for 12 h at 37℃. After completion of immunodiffusion, the gel was washed with 0.9% NaCl and stained with

Coomassie brilliant blue prepared in a mixture of distilled water/ethanol/acetic acid (9/9/2, v/v/v).

Results and Discussion

The crude enzyme extract of rice callus used in the present study contained six to eight POD isozymes designated as RC1, RC2, RC3, RA1, RA2, RA3 and RA4 when subjected to starch gel electrophoresis at pH 7.0 (Figure 1). Among them, fast- migrating cationic POD isozyme RC3 was purified from rice callus as shown in Figure 2. Initial elution of CM-cellulose column with 30 mM sodium phosphate buffer (pH 6.0) after absorbing the column with crude enzyme preparation yielded largely a mixture of anionic POD isozymes (RA1, RA2, RA3 and RA4) and cationic POD isozymes (RC1 and RC2). And then Sephacryl S-100 gel filtration fractions containing POD isozyme RC3 provided single polypeptide band in the SDS-polyacrylamide gel (Figure 3). Comparison of the relative electrophoretic mobility of the purified POD isozyme RC3 in the SDS-polyacrylamide gel indicated that the subunit molecular mass of RC3 was approximately 34 KDa (Figure 4). The native molecular mass of RC3 by the gel filtration was 38 KDa (Figure 4). These results suggest that

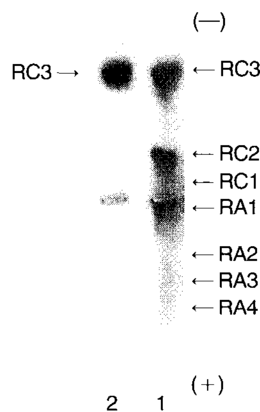

Figure 1. Starch gel electrophoregram of peroxidase (POD) isozymes from rice (*Oryza sativa* cv. Nak-dong) callus. Lane 1: crude extract, lane 2: fast-migrating cationic POD isozyme RC3.

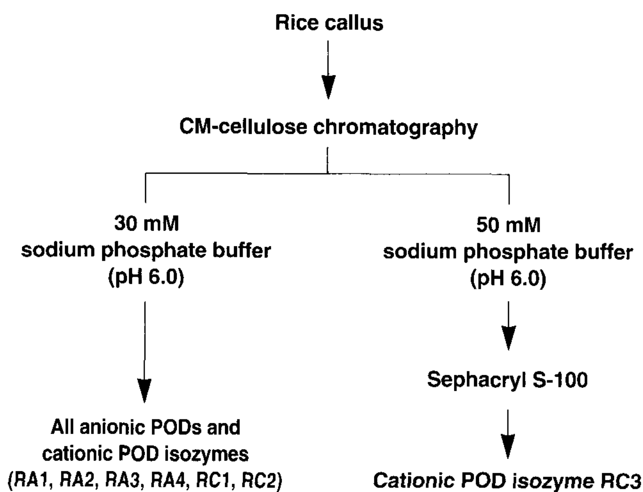

Figure 2. Purification scheme of fast-migrating peroxidase isozyme RC3 from rice callus (*Oryza sativa* cv. Nak-dong).

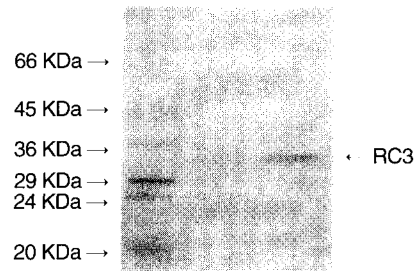

Figure 3. SDS-polyacrylamide gel electrophoregram of fast-migrating cationic peroxidase isozyme RC3 from rice callus.

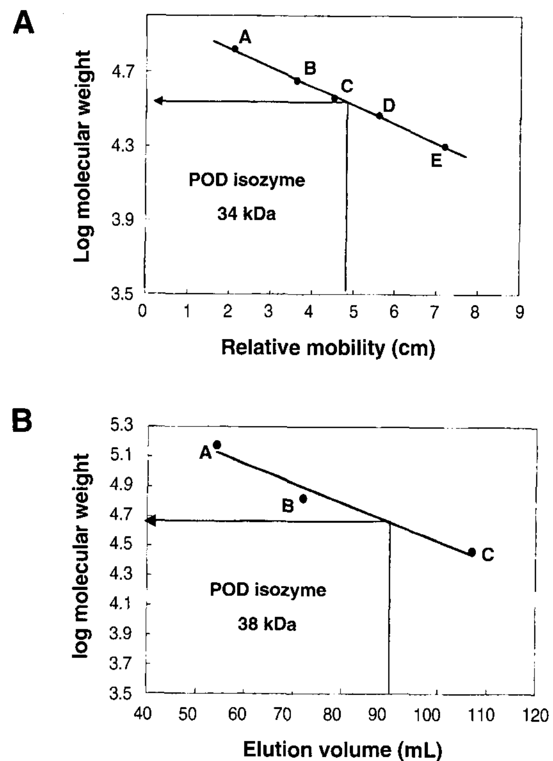

Figure 4. Determination of molecular mass of peroxidase isozyme RC3 by SDS-PAGE (A). Molecular mass markers are as follows. A: 66 KDa; B: 45 KDa; C: 36 KDa; D: 29 KDa; E: 20 KDa. Determination of molecular mass of RC3 by Sephacryl S-100 gel filtration(B). Molecular mass markers are as follows. A : 150 kDa; B: 66 kDa; C: 29 kDa.

this enzyme is composed of only one polypeptide and its molecular mass is smaller than those of other plant POD isozymes. Moreover, POD isozyme RC3 contained carbohydrate portion (data not shown) using PAS (Periodic Acid Schiff's reagent) stainig method described by Glossman and Neville (1971). POD isozymes from various plants also contained carbohydrate. The carbohydrate structures of Korean radish POD isozyme, A1, A2 and C3 were elucidated by N-glycan profiling, monosaccharide composition analysis and sequencing of major N-glycans (Kim and Kim, 1996). Only one potential N-glycosylation site with general sequence Asn-X-Thr/Ser was reported to be present in the deduced amino acid sequence of prxK1 cDNA from Korean radish (Park and Kim, 1996).

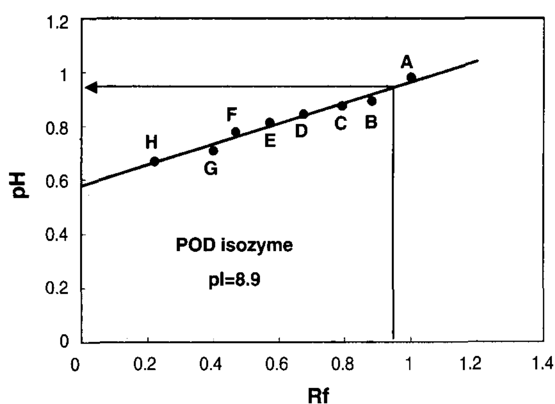

Figure 5. Determination of the isoelectric point of peroxidase isozyme RC3 by isoelectric focusing, The proteins, starting from the cathode, and their corresponding pI values are; A: cytochrome c (pI 9.6), B: lentil lectin (pI 7.8), C: human hemoglobin C (pI 7.5), D: equine myoglobin (pI 7.0), E: humancarbonic anhydrase (pI 6.5), F: bovine carbonic anhydrase (pI 6.0), G: β-lactoglobulin B (pI 5.1), and H: phycocyanin (pI 4.65).

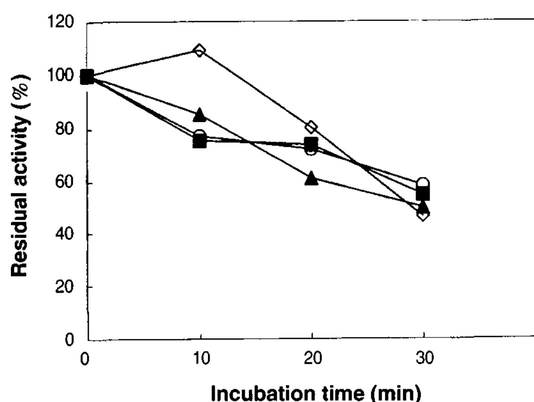

Figure 6. Effect of heat on the stability of peroxidase isozyme RC3. Enzyme solution was placed in thermostat at 20℃ (○), 30℃ (■), 40℃ (▲) and 50℃ (◇) for indicated heating time.

The pI value of RC3 was determined to be 8.9 (Figure 5). The pI value of the far migrating cationic POD isozyme C3 from Korean radish was reported to be 8.6 (Lee and Kim, 1994). Generally, plant PODs are known to be resistant to thermal inactivation (Welinder, 1985), and therefore they have been used as an indicator of blanching and other heat treatment. At room temperature, POD isozyme RC3 maintained its full activity for 30 min. However, when the enzyme was incubated at 40℃ for 30 min, about 50% of the activity was lost (Figure 6). It is unlikely that rice RC3 is thermostable. Ouchterlony double immunodiffusion using anti RC3 antiserum revealed that anti RC3 antiserum reacted with RC3 (Figure 7). In case of Korean radish POD isozymes, cationic isozyme C1 and C3 revealed an immunological identity to each other (Lee et al., 1994).

The optimum pHs and Km values of the enzyme against various substrates were determined (Table 1). POD

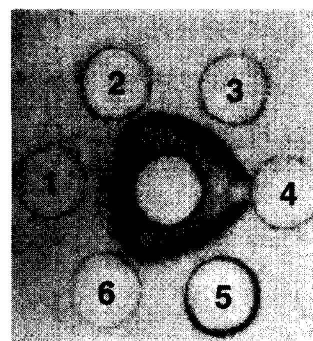

Figure 7. Ouchterlony double immunodiffusion analysis of peroxidase isozyme RC3. Center well: anti RC3 antiserum, well 1 , 3, 5: purified RC3, well 2, 4, 6: saline.

Table 1. Summary of Km values and pH optima toward various substrates of fast-migrating cationic peroxidases isozyme RC3 from rice callus.

Substrate	RC3	
	Optimum pH	Km (mM)
Guaiacol	6.0	10.5
H_2O_2	6.0	3.2
o-Dianisine	4.0	0.4
Esculetin	-	-
Caffeic acid	5.0	0.08
Ferulic acid	4.0	0.01
Ascorbic acid	7.0	0.01
Coniferyl alcohol	7.0	0.02

-: Not reactive

isozyme RC3 had pH optimum around 6.0 when guaiacol was used as a substrate, which is similar to that of other PODs. The optimum pHs against various substrates such as ferulic acid, coniferyl alcohol, o-dianisidine, ascorbic acid and caffeic acid were determined to be 4.0-7.0. POD isozyme RC3 had the Km value of 10.5 mM for guaiacol and 3.2 mM for H_2O_2. POD isozyme RC3 was found to have higher Km values for H_2O_2 as compared to other cationic POD isozymes from Korean radish, such as C1 and C3. H_2O_2 was known to be the major oxygen radical in the plant (Wise and Naylor, 1987) and it increased greatly under the cold injury (Prasad et al., 1994). Therefore, it is unlikely that RC3 with low affinity for H_2O_2 may play the role of H_2O_2 detoxification. When some naturally occurring phenolic substrates were tested (Lee and Kim, 1994), POD isozyme RC3 had very low Km vaule of 0.01 mM for ferulic acid and ascorbic acid. Considering the reports that ferulic acid was the intermediate of lignin biosynthesis in the cell wall, POD isozyme RC3 might be involved in the specific step of lignin biosynthesis. However, unlike other Korean radish POD isozymes, RC3 could not use esculetin as a substrate. Esculetin, known to be the naturally occurring growth regulator, was reported to be oxidized with low affinity for far migrating cationic POD isozyme C3 in Korean radish root (Lee and Kim, 1994). Therefore, POD isozyme RC3 seems to have unique functions in the lignin biosynthesis in terms of its special catalytic ability. More detailed catalytic data and structural studies are needed to investigate the characteristics of POD isozymes in rice cell.

Acknowledgement

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