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Characteristics of a Low Molecular Weight Minor Anionic Isoperoxidase A_{3n} from Radish

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A minor anionic isoperoxidase named A_{3n}, was isolated from Korean radish (*Raphanus sativus* L.) root. Purification of the enzyme was accomplished by CM-cellulose chromatography, DEAE-Sephacel chromatography, and Sephadex G-75 gel filtration. The enzyme was a glycoprotein with molecular weight of approximately 31,000 as determined by SDS-PAGE and 33,000 by Sepadex G-150 gel filtration, which is by far the smallest among the reported isoperoxidases. The pI value was 3.5. The optimum pH of the enzyme was 6.5 for guaiacol and H_2O_2, and the K_m values for guaiacol and H_2O_2 were 13.3 mM and 1.5 mM, respectively. Kinetic studies with various substrates revealed that only A_{3n}, unlike other isoperoxidases from radish, did not use scopoletin as a substrate and had very low K_m value of 0.25 mM for ferulic acid among naturally occurring phenolic substrates.

Keywords: Characteristics, Peroxidase, Radish (*Raphanus sativus* L.).

Introduction

Plants contain abundant amounts of peroxidases [EC 1.11.1.7] that exhibit broad substrate specificities (van Huystee and Cairn, 1982). Plant peroxidases are associated with the cell wall where they generate phenoxy compounds from cinamic acids and the phenoxy compounds then polymerize in a lignification process. In addition, plant peroxidases degrade indole-3-acetic acid. Therefore, they obviously play a key role in plant growth and development (van Huystee, 1987). Usually, isoperoxidases in a given species are separated into two distinct groups, viz., cationic and anionic isoperoxidases, according to the migration distances in starch gel electrophoresis (Kim *et al.*, 1980). There are notable differences in enzymatic properties between cationic and anionic peroxidases from Japanese radish and horseradish (Shih *et al.*, 1971; Welinder and Mazza, 1975). In Korean radish, there are at least eight isoperoxidases, and some of them have been isolated and their enzymatic properties studied on the protein level (Yoo and Kim, 1987; Lee and Kim, 1990; 1994; 1998; Lee *et al.*, 1994) and carbohydrate level (Kim and Kim, 1996). Moreover, the prxK1 gene encoding Korean radish isoperoxidase has been isolated from the genomic DNA library using a partial cDNA as a probe, and the nucleotide sequence of the genomic DNA was detemined. The deduced amino acid sequences and putative glycosylation site were also determined (Park and Kim, 1996a; 1996b).

The present study describes the purification and characterization of the minor anionic isoperoxidase named A_{3n}, from Korean radish root. Some of its kinetic and physicochemical properties have been investigated and compared with other isoperoxidases in detail.

Materials and Methods

Materials Fresh Korean radish (*Raphanus sativus* L.) roots were purchased from a local market near Shinchon, Seoul. The substrates for peroxidase, such as guaiacol, dianisidine and ferulic acid, were purchased from Sigma (St. Louis, USA). The resins for chromatography were purchased from Sigma (CM-cellulose and Sephadex G-75) and Pharmacia (DEAE-Sephacel).

Peroxidase assays with various substrates The peroxidase activity with guaiacol as a substrate was assayed by a modified procedure of Kim *et al.* (1980). The assay mixture contained 40 mM phosphate buffer, 15 mM guaiacol, 5 mM H_2O_2 and 50 μl 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 the Kontron UV/VIS spectrophotometer 930. Typical assay conditions and wavelengths for

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scopoletion, *o*-dianisidine, ferulic acid, esculetion, and chlorogenic acid were determined according to the method of Kim *et al.* (1980).

Preparation of crude enzyme Korean radish root homogenate in 5 mM sodium phosphate buffer (pH 6.0) was filtered through four layers of gauze and clarified by centrifugation. The supernatant was adjusted to 80% saturation with $(NH_4)_2SO_4$. The pellet from $(NH_4)_2SO_4$ treatment was then dissolved in a minimun volume of 5 mM sodium phosphate buffer (pH 6.0) and dialyzed against the same buffer.

Gel electrophoresis Starch gel electrophoresis was performed as described by Kim *et al.* (1980). Isoperoxidase 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. SDS-polyacrylamide gel eletrophoresis was carried out as described by Laemmli (1970).

Enzyme purification The crude enzyme preparation was loaded on a CM-cellulose ion exchange column (3.5 × 12 cm) preequilibrated with 5 mM sodium phosphate buffer (pH 6.0). The column was washed with the same buffer until the absorbance of the eluant containing all anionic isoperoxidases at 280 nm became zero. The flow rate was adjusted to 20 ml/h and 10 ml fractions were collected. The fractions containing four anionic isoperoxidases, A_1, A_2, A_{3n}, and A_3, were collected and concentrated with Amicon ultrafiltration membrane PM10. The concentrated enzyme fractions were dialyzed against 1 mM sodium phosphate buffer (pH 6.0) overnight. The dialyzed sample was applied on a DEAE-Sephacel ion exchange column (2.5 × 4 cm) preequilibrated with 1 mM sodium phosphate buffer. Isoperoxidase A_1 was eluted with 1 mM sodium phosphate buffer followed by isoperoxidase A_{3n} isocratically. The fractions containing isoperoxidase A_{3n} were collected and lyophilized. The lyophilized sample was dialyzed against 50 mM sodium phosphate buffer (pH 6.0) and then applied to a Sephadex G-75 column (1.3 × 110 cm) preequilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was eluted with the same buffer at a flow rate of 8 ml/h and 3 ml fractions were pooled. The purified enzyme solution thus obtained was collected and used for all other studies.

Molecular weight determination The molecular weight of the isoperoxidase A_{3n} was estimated by gel filtration chromatography on Sephadex G-150 column (1.5 × 78 cm) according to the procedure of Andrews (1964). The standard proteins were globulin (150,000), bovine serum albumin (66,000), and myoglobin (18,000). The subunit molecular weight of the enzyme was determined by SDS-polyacrylamide gel electrophoresis. The gel consisted of 12.5% acrylamide running gel and 3% acrylamide stacking gel. The standard proteins used were phosphorylase b (96,000), egg. albumin (45,000), carbonic anhydrase (31,000), and trypsin inhibitor (20,000).

Determination of glycosylation The procedure described was used with the Schiff's reagent as a visualizing reagent (Grossman and Neville, 1971).

Determination of the isoelectric point of isoperoxidase A_{3n} The isoelectric point (pI) of A_{3n} was determined by the modified method of Winter *et al.* (1977). A suitable amount of samples was applied on the polyacrylamide gel plate containing ampholine carrier-ampholite (pH 3.5–9.0).

Heat stability of isoperoxidase A_{3n} Isoperoxidase A_{3n} was incubated in thermostats at 25°C, 35°C, 45°C, and 55°C for various incubation times to investigate the heat stability of the enzyme.

Effect of organic solvent on the isoperoxidase A_{3n} activity The effects of various alcohols such as *t*-butanol, *n*-propanol, ethanol, and methanol on the isoperoxidase A_{3n} activity were studied. The enzyme was incubated for 3 min in the presence of several alcohols, and the residual enzyme activity was measured.

Ouchterlony double immunodiffusion Immunodiffusion was performed at room temperature in a 1% agarose gel, according to method of Ouchterlony and Nilsson (1973). Proper amounts of isoperoxidase A_{3n} were incubated with antiserum C_3, A_1, and A_2 for 12 h at 37°C. After completion of immunodiffusion, the gel was washed with 0.9% NaCl and stained with Coomassie Brilliant Blue.

Results and Discussion

Enzyme purification The crude enzyme extract of Korean radish root used in the present study contained six to eight isoperoxidases designated as C_1, C_2, C_3, A_1, A_2, A_{3n}, and A_3 when subjected to starch gel electrophoresis at pH 7.0 (data not shown). Among them, major cationic isoperoxidases C_3 and anionic isoperoxidase A_1, A_2, and A_3 were purified and their enzymatic and immunological properties were characterized (Lee and Kim, 1994; Lee *et al.*, 1994). However, the purification of minor isoperoxidase was very difficult because of its low content in the cell. Initial elution of the CM-cellulose column with 5 mM sodium phosphate buffer (pH 6.0), after absorbing the column with crude enzyme preparation, largely yielded a mixture of anionic isoperoxidases (A_1, A_2, A_{3n}, and A_3), which could be separated subsequently by chromatography on a DEAE-Sephacel column (Lee and Kim, 1994).

The isoperoxidase A_{3n} was separated from other isoperoxidases by isocratic elution of the DEAE-Sephacel column with 1 mM sodium phosphate buffer (pH 6.0) after eluting isoperoxidase A_1 as indicated in Fig. 1B. Figure 1C shows a Sephadex G-75 gel filtration of DEAE-Sephacel fractions which contain isoperoxidase A_{3n} activity. This procedure removed minor contaminating proteins and provided a single polypeptide band in the SDS-polyacrylamide gel (Fig.2).

Molecular weight determination Comparison of the relative electrophoretic mobility of the purified isoperoxidase A_{3n} in the SDS-polyacrylamide gel with a set

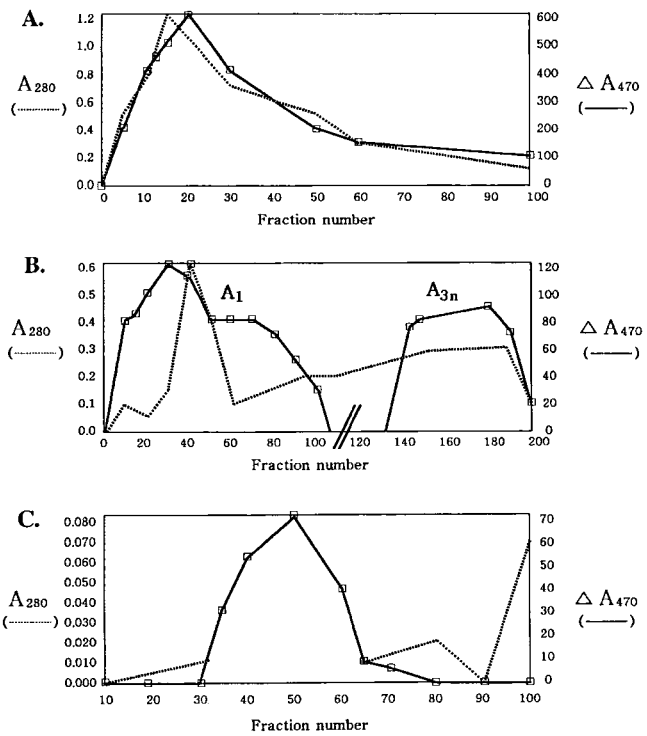

Fig. 1 Chromatographic purification of isoperoxidase A_{3n} from Korean radish root. A. CM-cellulose ion exchange choromatography. B. DEAE-Sephacel ion exchange chromatography for the separation of isoperoxidase A_{3n}. C. Sephadex G-75 gel filtration chromatography for the purification of isoperoxidase A_{3n}.

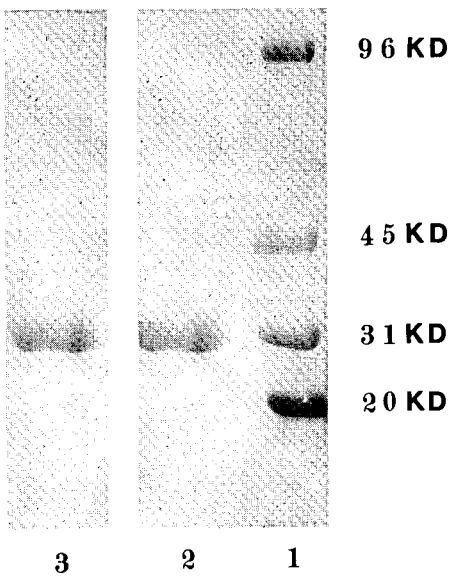

Fig. 2 SDS-polyacrylamide gel electrophoregram of isoperoxidase A_{3n} from Korean radish. The enzyme was stained with Coomassie Brilliant Blue R-250 for protein portion (lane 2) and glycoprotein staining reagent for carbohydrate portion (lane 3).

of proteins of known molecular weight indicated that the subunit molecular weight of A_{3n} from Korean radish root was approximately 31,000 (Fig. 2). The native molecular weigt of A_{3n} by the gel filtration of Sephadex G-150 was also 33,000 (Fig. 3). These results suggest that this enzyme is composed of only one polypeptide and its molecular weight is smaller to that of other isoperoxidases such as A_1, A_2, A_3, and C_3, which were reported to be 44,000, 43,700, 50,000, and 44,000, respectively (Yoo and Kim, 1987; Choi *et al.*, 1989).

Determination of glycosylation Using the PAS (Periodic Acid Shiff's reagent) staining method described by Glossman and Neville (1971), isoperoxidase A_{3n} with small molecular weight was shown to contain a carbohydrate portion (Fig. 2). Other isoperoxidases such as A_1, A_2, A_3, and C_3 also contain carbohydrate. The carbohydrate structures of two anionic peroxidase, A_1 and A_2, and one cationic peroxidase, C_3, 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 $prxK_1$ cDNA from Korean radish (Park and Kim, 1996a; 1996b), which is less than those of peroxidases from horseradish having eight N-glycosylation sites and of peanut which has five N-glycosylation sites (Welinder, 1979).

Isoelectric point determination The pI value of isoperoxidase A_{3n} was determined to be 3.5 (Fig. 4). That of the far migrating anionic isoperoxidase A3 was reported to be 3.0 (Lee and Kim, 1990). The pI values of isoperoxidase A_1, A_2, C_1, and C_3 were 4.0, 4.0, 8.6, and 9.0, respectively.

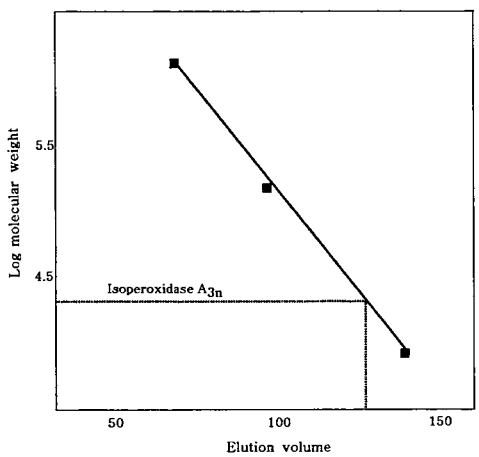

Fig. 3 Determination of native molecular weight of isoperoxidase A_{3n} by Sephadex G-150 gel filtration. The molecular weight markers were as follow; γ-globulin, 150 kDa; bovine serum albumin, 66 kDa; myoglobin, 18 kDa.

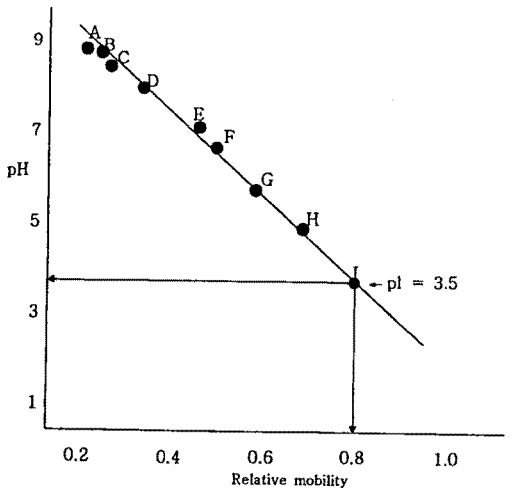

Fig. 4 Determination of the isoelectric point of isoperoxidase A_{3n} by isoelectric focusing. The proteins, starting from the cathode, and their corresponding pI values are: A. Lentil lectin basic band (pI 8.45); B. Lentil lectin middle band (pI 8.45); C. Lentil lectin acidic band (pI 8.15); D. Myoglobin basic band (pI 7.35); E. Myoglobin acidic band (pI 6.85); F. Human carbonic anhydrase B (pI 6.55); G. Y-lactoglobulin A (pI 5.20); H. Soybean trypsin inhibitor (pI 4.55); I. Amyloglucosidase (pI 3.5).

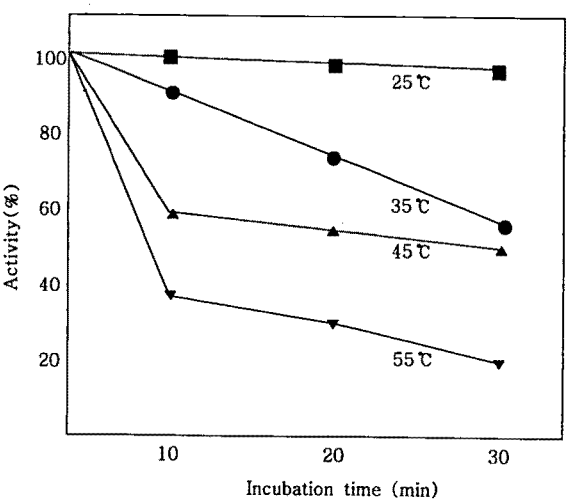

Fig. 5 Heat stability of isoperoxidase A_{3n} from Korean radish root. The enzyme solution was incubated in thermostat at 25°C (■), 35°C (●), 45°C (▲), and 55°C (▼) for various incubation times.

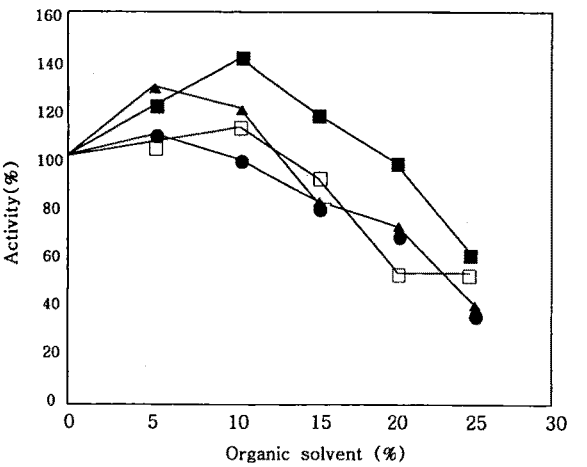

Fig. 6 Effect of various alcohols on isoperoxidase A_{3n} activity. The enzyme was incubated at 25°C for 3 min in the presence of alcohol, and the residual enzyme activity was measured. (■) *t*-butanol; (▲) *n*-propanol; (□) ethanol; (●) methanol.

Heat stability of isoperoxidase A_{3n} Generally, plant peroxidases are known to be resistant to thermal inactivation (Welinder, 1985), and therefore have been used as an indicator of blanching and other heat treatments. At room temperature, isoperoxidase A_{3n} maintained its full activity for 30 min and above. However, when the enzyme was incubated at 45°C for 10 min, about 50% of the activity was lost (Fig. 5). The heat stability of the enzyme was thought to be mainly due to the presence of disulfide bridges. It was reported that there are two possible disulfide bridges in the deduced amino acids sequence of prxK1 cDNA which may represent a basic isozyme (C_1, C_2, C_3, or C_{3n}) (Park and Kim, 1996a; 1996b).

Effect of organic solvent on the peroxidase activity In an attempt to investigate the protective effect of various alcohols as found in isoperoxidase A_1 (Lee and Kim, 1993), the effect of various alcohols on the isoperoxidase A_{3n} activity was studied (Fig. 6). When *t*-butanol ranging from 5 to 20% was incubated with the enzyme, the enzyme activity was increased between 20 to 40%; however, the enzyme activity was decreased approximately 40% in the presence of 25% of *t*-butanol. The enzyme activity also started to decrease in the presence of 15% of 1-propanol, 10% of ethanol, and 10% of methanol. These results suggest that the longer chain alcohols are better stabilizers of peroxidase activity than the short chain alcohols.

Immunological relationships of Korean radish isoperoxidases Ouchterlony double immunodiffusion experiment using antiserum C_3, A_1, and A_2 revealed that both antiserum A_1 and A_2, as well as antiserum C_3, did not cross-react with isoperoxidase A_{3n} (data not shown). Thus, isoperoxidase A_{3n} seems to have different antigenic determinants from C_3, A_1, and A_2. In our previous experiment, cationic isoperoxidase C_1 and C_3 revealed an immunological identity to each other, while these two isoperoxidases showed no immunological relationships with anionic isoperoxidases. Anionic isoperoxidases A_1 and A_2 showed partial identity to each other; however, these two isozymes did not cross-react with other anionic isoperoxidases (Lee *et al.*, 1994). These results suggest an

Table 1. Summary of K_m and pH optima for various synthetic and natural substrates of Korean radish peroxidases.

	optimum pH						K_m (mM)					
	C_1[a]	C_3[a]	A_1[b]	A_2[c]	A_{3n}	A_3[b]	C_1	C_3	A_1	A_2	A_{3n}	A_3
guaiacol	5.5	6.0	6.5	5.0	6.5	6.0	19.4	5.6	11	6.7	13.3	8
dianisidine	4.5	5.0	4.5	4.5	4.5	5.0	0.81	1.2	0.6	0.63	0.7	0.6
H_2O_2	5.5	5.0	6.5	5.0	6.5	6.0	0.19	0.77	2.5	1.38	1.5	1.0
scopoletin	4.5	5.0	6.0	4.0	–	4.5	0.91	5.6	0.24	0.18	–	1.67
ferulic acid	4.5	4.5	5.0	3.5	4.0	3.5	1.78	7.7	0.1	0.67	0.25	0.63
caffeic acid	6.0	N.D.	4.5	4.0	4.5	4.0	0.6	N.D.	1.54	1.43	1.4	2.5
esculetin	6.5	5.5	N.D.	5.5	6.0	6.0	0.71	4.8	N.D.	5.5	1.6	0.9
chlorogenic acid	5.5	N.D.	N.D.	5.0	5.0	4.0	0.38	N.D.	N.D.	1.5	1.25	1.4

N.D.: Not determined
a,b,c: Data were taken from Choi *et al.* (1989), Lee *et al.* (1994), and Yoo and Kim (1987), respectively.

immunological uniqueness of isoperoxidase A_{3n} among radish isoperoxidases.

Kinetic studies The effect of pH on the enzymatic activity of A_{3n} was determined using 40 mM sodium phosphate buffer, 40 mM sodium citrate buffer, and 40 mM sodium acetate buffer depending upon the pH range studied. Isoperoxidase A_{3n} had its pH optimum at around 6.5 when guaiacol was used as a substrate, which is similar to that of A_1. The optimum pHs against various substrates such as ferulic acid, esculetin, *o*-dianisidine, and caffeic acid were determined to be in the range of 4.0–6.5 (Table 1).

Isoperoxidase A_{3n} had a K_m value of 13.3 mM for guaiacol and 1.5 mM for H_2O_2. A_{3n} had higher K_m values for guaiacol when compared with those of the other major isoperoxidases C_3, A_1, A_2, and A_3. Isoperoxidase A_{3n}, along with other anionic isoperoxidases, was found to have a higher K_m value for H_2O_2 as compared to cationic isoperoxidases such as C_1 and C_3. H_2O_2 is known to be the major oxygen radical in the plant (Wise and Naylor, 1987) and it increases greatly under cold injury (Prasad *et al.*, 1994). Therefore, it is unlikely that anionic isoperoxidases 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), isoperoxidase A_{3n} had a very low K_m value (0.25 mM) for ferulic acid, unlike other isoperoxidases (C_1, C_3, A_2, and A_3). Considering the reports that ferulic acid was the intermediate of lignin biosynthesis in the cell wall and the main isoperoxidases located in the cell wall were anionic isoperoxidases, isoperoxidase A_{3n} along with other anionic isoperoxidases might be involved in the specific step of lignin biosynthesis. However, unlike other Korean radish isoperoxidases, only A_{3n} could not use scopoletin as a substrate. Scopoletin, known to be the naturally occurring growth regulator, was reported to be oxidized in a complex multistep mechanism, resulting in the sigmoidal saturation curve as shown in C_1 and A_3 (Choi *et al.*, 1989; Lee and Kim, 1990). Therefore, isoperoxidase A_{3n} 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 determine the real role of peroxidase isozymes in physiological processes.

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