Identification and Molecular Characterization of Novel Peroxidase with Structural Protein-like Properties

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Elicitor treatment or mechanical damage to Scutellaria baicalensis Georgi (skullcap plants) callus causes an immediate insolubilization of a 36-kDa protein into cell walls. The 36-kDa protein was identified as peroxidase 1 by analysis of its internal amino acid sequence and by immunoblotting using affinity-purified anti-peroxidase 1. Insolubilized peroxidase 1 is cross-linked to lignin through covalent bonds, and the cross-linking is catalyzed in the presence of H₂O₂ by peroxidase 1 itself. The properties of insolubilized peroxidase 1 resemble those of defense-related structural proteins (extensins and proline-rich proteins) cross-linked to cell wall. Although the isozymes peroxidases 2 and 3 have enzyme activities similar to peroxidase 1, they are not insolubilized by stress treatment. Molecular characterization established that peroxidase 1 contains regions characteristic of structural proteins, but peroxidases 2 and 3 do not have such regions. These results suggest that among the three isozymes, only peroxidase 1 has a structural protein-like function as well as an enzymatic function.

Most higher plants can quickly activate various defense systems to protect themselves from pathogen attack, injury, or other forms of stress. Among them, insolubilization of structural proteins has been believed to strengthen the cell wall barrier against pathogen attack (1). Extensins and proline-rich proteins (PRPs) that act as defense-related structural proteins have been identified in both dicotyledons and monocotyledons (2). Extensins and PRPs have been thought to be nonenzymatic and purely structural proteins, and molecular characterization of these proteins has established that they all possess several structural characteristics in common: 1) they are basic; 2) they are abundant in hydroxyproline, proline, lysine, and tyrosine residues; 3) and they have highly repetitive peptide sequence with characteristic motifs (e.g. Ser-Hyp-Hyp-Hyp-Hyp for extensins, and Pro-Pro-Val-Tyr-Lys for PRPs) (2–4). Their insolubilization mechanisms have been also well examined, confirming that insolubilization of both structural proteins, which involves H₂O₂-mediated oxidative cross-linking to cell walls, is catalyzed by coexisting peroxidases (5–7).

Peroxidases are ubiquitous enzymes that catalyze oxidation of cellular components in the presence of H₂O₂. Most higher plants contain a number of peroxidase isozymes, which can be classified into two (anionic and cationic) or three (anionic, neutral, and cationic) subgroups according to their isoelectric focusing mobilities, and these isozymes exist in cytosol, chloroplast, vacuole, and cell wall (8, 9). Their physiological roles have been extensively investigated, and it has been demonstrated that they catalyze a variety of important reactions, such as indole-3-acetic acid catabolism (10), lignin biosynthesis (11, 12), suberization of cell wall (13), and detoxification of H₂O₂ (9, 14). These peroxidases are soluble in buffers containing detergents or high concentration of salt, whereas peroxidases that cannot be extracted using these buffers are also found in various plants (15–18). Although the latter peroxidases have been shown to be covalently bound to cell walls, almost nothing is known about their physiological importance, their structural characteristics, or the mechanism of covalent bond formation.

Previously, we identified a novel H₂O₂-detoxyifying system induced by elicitor treatment in Scutellaria cells and demonstrated that two ionically bound wall peroxidases (peroxidases 1 and 2) effectively metabolize large amounts of H₂O₂ produced during oxidative burst (19). Further studies have revealed that peroxidase 1 possesses quite novel functions; like extensins and PRPs, peroxidase 1 is covalently bound to cell wall in response to elicitor treatment and mechanical damage, and surprisingly, this reaction is catalyzed by the action of peroxidase 1 itself in the presence of H₂O₂. We report here the novel properties and possible physiological roles of peroxidase 1. In addition, we have found that the isozymes peroxidase 2 and the newly identified peroxidase 3 show enzymatic properties similar to peroxidase 1, but they do not become insoluble after stress treatment. Because the divergent and shared properties of these peroxidases are assumed to arise from their structures, we have isolated cDNA clones encoding peroxidases 1–3 and predicted their amino acid sequences. We also describe the primary structures of these Scutellaria peroxidases.

EXPERIMENTAL PROCEDURES

Stress Treatment of Scutellaria Calluses—The four-week-old calluses of Scutellaria baicalensis (5 g), which was cultured on Murashige-Skoog medium (21) as described previously (20), were subdivided into 100 ml flasks, and 20 ml of liquid Murashige-Skoog medium was added to each flask. After 200 µl of 10% (v/v) yeast extract dissolved in the liquid Murashige-Skoog medium was added (final concentration of yeast extract, 0.1%), the calluses were incubated at 25 °C for 3 h. Treatment of the callus with H₂O₂ was conducted as described above, except that 100 µl of 1 m H₂O₂ (final H₂O₂ concentration, 5 mM) was used instead of the yeast extract solution. Mechanical damage to callus was done as follows. After an aliquot (0.5 g) of the callus was homogenized with 20 ml

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank® TM/EBI Data Bank with accession number(s) AB024437, AB024438, and AB024439 for peroxidases 1–3, respectively.

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‡ The abbreviations used are: PRP, proline-rich protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; RACE, rapid amplification of the cDNA ends; PCR, polymerase chain reaction; bp, base pair(s).
of the liquid Murashige-Skoog medium, the intact cells (4.5 g) was added to the homogenate and incubated as above.

**Immunological Procedures**—Polyclonal antisera against peroxidase 1 was generated in white female rabbits using 75 µg of purified peroxidase 1 per injection. The antiserum obtained after the second booster was diluted in 100 µl of an affinity purification buffer and applied to CNBr-activated Sepharose 4B as described by the manufacturer (Amersham Pharmacia Biotech). Anti-peroxidase 1 was eluted with 2 M MgCl2. For Western blotting analysis, samples were subjected to SDS-PAGE (12.5% acrylamide gels) and transferred to polyvinylidene difluoride membranes as above. The fragments were NH2-terminally sequenced on an Applied Biosystems 473A protein sequencer.

**Preparation and Fractionation of Cell Walls from Scutellaria Callus**—To avoid insolubilization of peroxidase 1 during preparation of cell wall, ionically bound wall proteins were removed by washing the 4-week-old callus (100 g) with 1 M NaCl (2,000 ml). The NaCl-washed calluses were homogenized with 50 ml of 50 mM phosphate buffer (pH 7.5), 0.5% (w/v) Triton X-100 and was used as crude cell wall. Fractionation and purification of peroxidase 1 was eluted at a flow rate of 1 ml/min by a linear gradient of NaCl (0–0.2 M). The effluent was microsequenced on an Applied Biosystems 473A protein sequencer.

**Immunostaining of crude cell wall** was conducted as follows. Crude cell wall, which was described as above, was incu-bated at room temperature for 1 h in blocking buffer containing affinity-purified anti-peroxidase 1 diluted in blocking buffer. Antibody binding was visualized using horseradish peroxidase-conjugated secondary antibodies (Wako, Tokyo) and 1-chrolo-4-naphtol (Sigma).

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1 min at 94, 55 and 72 °C. 5'-RACE product was created by first performing PCR with gene-specific primer 1 and adapter primers 1 and 3 using template cDNA pool 4 (5 cycles of 1 min at 94, 55, and 72 °C, then 30 cycles of 1 min at 94, 55, and 72 °C). Then a second round of PCR (30 cycles of 1 min at 94, 55, and 72 °C) with gene-specific primer 7, adapter primer 1, and first PCR products yielded a 5'-RACE product (350 bp). Sequencing of the amplified fragments was carried out as above. Concerning the amplification and sequencing of cDNA encoding peroxidase 3, the same PCR conditions were employed, except that degenerate primer 4 and gene-specific primers 8, 9, and 10 were used instead of degenerate primer 3 and gene-specific primers 5, 6, and 7, respectively.

RESULTS

Identification of a Cell Wall Protein Insolubilized in Response to Elicitor Treatment—Previously, Bradley et al. (1) reported that treatment of French bean (Phaseolus vulgaris L.) or soybean (Glycine max L.) cells with elicitor causes rapid insolubilization of extensins and PRPs in cell walls. Therefore, we investigated whether similar treatment affects extractability of structural proteins in S. baicalensis. After Scutellaria calluses were treated with yeast extract as an elicitor, cell wall proteins were extracted with NaCl and then analyzed by SDS-PAGE. When NaCl-extractable proteins in the elicitor-treated callus were compared with those in the control callus, apparent decreases in a 36-kDa protein were observed in the former callus (Fig. 1A). To obtain structural information on this 36-kDa protein, cell wall proteins resolved by SDS-PAGE were electroblotted onto polyvinylidene difluoride membranes, and the 36-kDa protein was NH₂-terminally sequenced. However, sequencing was unsuccessful because the NH₂ terminus appeared to be chemically blocked. The 36-kDa protein was eluted from the gel and digested using endoproteinase Glu-C. NH₂ terminus sequencing of the hydrolysates (1.2- and 1.6-kDa fragments) surprisingly revealed that both fragments contain motifs very similar to the peroxidase active site (Ala-Ala-Gly-Leu-Ile-Arg-Leu-His-Phe-Asp) and the ligand of heme (Met-Val-Thr-Ile-Ser-Gly-Ala-His-Thr-Leu), respectively (8). However, the motifs characteristic of extensins and PRPs were not found. Previously, we identified two cationic peroxidase isozymes (peroxidases 1 and 2) catalyzing H₂O₂-metabolism in S. baicalensis (19), and the molecular mass of the 36-kDa protein was closer to peroxidase 1 than peroxidase 2. Finally, the 36-kDa protein was identified as peroxidase 1 by Western blotting using affinity-purified anti-peroxidase 1 antibody (Fig. 1B).

In situ immunostaining of Scutellaria callus was carried out with anti-peroxidase 1 antibody by a method described previously (1), but it did not differentiate between control and elicitor-treated calluses (data not shown). We hypothesized that ionically bound wall peroxidase 1 is not completely removed using this staining method, and therefore, soluble peroxidase-free cell wall fractions, which were prepared by extensive washing the callus with NaCl, were used for immunostaining. Consequently, the immunoreactivity in the isolated cell walls was significantly enhanced by elicitor treatment (Fig. 2), indicating that the decrease in NaCl-extractable peroxidase 1 is due to insolubilization in the cell walls. Although we attempted to extract insolubilized peroxidase 1 from the elicited callus using various methods including SDS extraction (1), acidified cholate treatment (24), and limited proteolysis (25), peroxidase 1 could not be solubilized. This observation, taken together with the results of immunostaining, suggests that peroxidase 1 is rigidly cross-linked to cell wall through covalent bonds.

Mechanism of Peroxidase Insolubilization—Fig. 1A shows that mechanical damage also induces similar decrease in NaCl-extractable peroxidase 1. Because elicitor treatment or mechanical damage causes rapid production of H₂O₂ in plant cells, we assumed that H₂O₂ is indispensable for the insolubilization of peroxidase. This assumption was strengthened by the fact that treatment of Scutellaria callus with H₂O₂ promotes decreases in NaCl-extractable peroxidase 1 by Western blotting. As shown in Fig. 3A, insolubilization of peroxidase 1 was found only in the presence of crude cell wall and H₂O₂. These results indicated that, like extensins and PRPs, insolubilization of peroxidase 1 involves H₂O₂-dependent cross-linking to cell walls. Peroxidase 1 was thought to catalyze the reaction itself because the insolubilization never occurred using inactivated peroxidase 1 (Fig. 3B). Brownleaden et al. (7) demonstrated polymerization of extensins by in vitro peroxidase reaction in the absence of cell walls, whereas peroxidase 1 was not polymerized under similar conditions.

The crude cell walls were further fractionated into pectin,
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**Fig. 2.** Immunostaining of cell walls. After cell walls prepared from control and elicitor-treated callus were incubated with affinity-purified anti-peroxidase 1 and then with alkaline phosphatase-conjugated secondary antibodies, antibody binding was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (samples 2 and 3, respectively). Sample 1 was the control cell wall stained by similar procedures in the absence of anti-peroxidase 1.

**Fig. 3.** In vitro insolubilization of peroxidase 1. A, conditions required for cross-linking of peroxidase 1. Purified peroxidase 1 (2 μg) dissolved in 200 μl of 10 mM citrate buffer (pH 4.0) was incubated at 25 °C for 2 h in the presence of H₂O₂ (final concentration, 5 mM) and/or crude cell wall (5 mg). After 1 M NaCl (5 ml) was added to each sample, NaCl extracts were analyzed by Western blotting. B, effect of peroxidase activity on insolubilization. Intact peroxidase 1 or inactivated peroxidase 1 (each 2 μg) was incubated at 25 °C for 2 h in 200 μl of the above citrate buffer containing 5 mM H₂O₂ and crude cell wall (5 mg). Peroxidase 1 was extracted with 1 M NaCl and analyzed by Western blotting as above. C, binding ability of peroxidase 1 to various cell wall components. Purified peroxidase 1 (2 μg) was dissolved in 200 μl of the above citrate buffer containing 5 mM H₂O₂. The peroxidase solution was incubated at 25 °C for 2 h in the absence (blank) or presence (each 5 mg) of isolated cell wall component (pectin, hemicellulose, or lignocellulose). NaCl-soluble peroxidase 1 was analyzed by Western blotting as above.

**Fig. 4.** Kinetics of elicitor-stimulated insolubilization of peroxidase 1. The calluses (1 g) were incubated with 4 ml of 0.1% yeast extract or 5 mM H₂O₂ at 25 °C for different times (0–180 min). Ionically bound wall proteins were extracted with 1 M NaCl (20 ml), dialyzed against H₂O₂, and lyophilized. The lyophilized samples were resolved by SDS-PAGE (12.5% acrylamide gels). After the gel was stained with Coomasie Brilliant Blue, the intensity of peroxidase 1 was estimated using an image scanner. The S.D. was always within 5% of the mean of triplicate determinations.

hemicellulose, and lignocellulose (23), and binding of peroxidase 1 to each fraction was tested. Fig. 3C shows that peroxidase 1 bind only to lignocellulose. This lignocellulose fraction from the elicited callus was treated with cellulase and anhydrous hydrofluoric acid, which catalyze cleavage of glycosidic bonds, but treatment using both reagents did not solubilize peroxidase 1, confirming that peroxidase 1 is not bound to the cellulose moiety (data not shown).

**Kinetics of Insolubilization.** The kinetics of peroxidase 1 insolubilization were determined by monitoring the band intensity of peroxidase 1 by SDS-PAGE (Fig. 4). NaCl-soluble peroxidase 1 quickly decreased until 30 min after addition of elicitor and thereafter slowly insolubilized. More than 80% of ionically bound wall peroxidase 1 was cross-linked to the cell walls at 60 min after elicitation. Its significant decrease was not detectable after 180 min. The insolubilization of extensins and PRPs, which is initiated within 2–5 min and completed within 20–30 min, is one of the earliest defense responses (1), whereas the insolubilization of peroxidase 1 was somewhat slower. We hypothesized that this lower rate is due to scarcity of H₂O₂, because Scutellaria cells rapidly metabolize large amounts of H₂O₂ produced during oxidative burst (19). Hence, we conducted experiments using an excess of H₂O₂, but only a slight increase in the insolubilization rate occurred (Fig. 4).

**Properties of Other Peroxidase Isozymes.** Our previous studies demonstrated the presence of other isozymes in S. baicalensis during purification of peroxidases 1 and 2 (19), but their properties were not examined. To precisely compare the properties of these isozymes with those of peroxidase 1, we attempted purification of them. A cell wall protein fraction, which was prepared from 4-week-old callus as described previously (19), was applied to a CM-cellulose (CM-52) column and then to a hydroxyapatite column to isolate a new isozyme (peroxidase 3), together with peroxidases 1 and 2. The peroxidase-3-containing fractions were further chromatographed over a CM-52 column, purifying peroxidase 3 to homogeneity (Fig. 5).

SDS-PAGE of peroxidase 3 revealed that it has the same molecular mass (34 kDa) as peroxidase 2 (Fig. 5), whereas its pl value (8.5) was cationic, like peroxidases 1 and 2. Peroxidase 3 catalyzed degradation of the flavones baicalein and luteolin, but the other flavones (baicalin, apigenin, oroxylin A, and wogonin) were not substrates of this isozyme. This catalytic ability was identical to that of peroxidases 1 and 2 (19). In the presence of baicalein, its H₂O₂-metabolizing activity (1.29 μkat/mol of protein) also resembled those of peroxidases 1 and 2 (1.38 μkat/mol of protein). Thus, enzymatic properties of these three peroxidases were similar, although the responses of peroxidases 2 and 3 to oxidative stress were apparently different from that of peroxidase 1. The intensity of a 34-kDa band corresponding to peroxidases 2 and 3 was not significantly decreased by elicitor treatment or mechanical damage, contrary to that of peroxidase 1 (Fig. 1A). Furthermore, we evaluated changes in ionically bound wall peroxidases 2 and 3 levels by HPLC. Fig. 6 demonstrates that the levels of NaCl-soluble peroxidases 2 and 3 are not affected by elicitor treatment.

**Molecular Characterization of Peroxidases 1–3.** We next analyzed the amino acid contents of all isozymes. Although extensins and PRPs are characterized by high (hydroxyl)proline
and lysine contents (2), peroxidases 1–3 had much lower proline (5.1, 4.3, and 4.0%, respectively) and lysine (2.9, 2.5, and 2.6%, respectively) contents versus 25% hydroxyproline, 15% proline, and 14% lysine for maize extensin (26). None of the peroxidases contained hydroxyproline residues. As amino acid analyses did not provide useful findings to explain differences in properties of these peroxidases, we isolated and sequenced cDNA clones coding for them to reveal their primary structures. A reverse transcription-PCR method was employed to isolate the genes encoding peroxidases. Purified peroxidases were treated with endoproteinase Glu-C and cyanogen bromide, and the resulting fragments were NH$_2$-terminally sequenced. Internal cDNA fragments were amplified using degenerate oligonucleotide primers, which were designed based on the amino acid sequences. Moreover, cDNA fragments containing 3'-end and 5'-end regions were obtained by 3'- and 5'-RACE. Finally, the cDNA fragments, which were assumed to possess the entire coding region, were amplified using gene-specific primers designed from 3'- and 5'-RACE products. Nucleotide sequences of the amplified fragments were determined from both strands to ensure accuracy.

The cloned genes encoding peroxidases 1–3 consisted of 966-, 975-, and 954-nucleotide open reading frames encoding 322, 325 and 318 amino acids, respectively. The PSORT program predicted that 19, 28, and 24 amino acid residues from each first methionine, which are shaded in yellow in Fig. 7, are signal peptides, thus suggesting that mature peroxidases 1–3 consist of 303, 297, and 294 amino acid residues, respectively. We confirmed that each deduced amino acid sequence contains all fragments obtained by digestion with endoproteinase Glu-C or cyanogen bromide. Mature peroxidase 1 showed 47 and 42% identity with mature peroxidases 2 and 3, respectively, whereas mature peroxidase 2 displayed 59% identity with mature peroxidase 3.

All peroxidases possess several highly homologous regions including the peroxidase active site (Fig. 7, shaded in pink) and the ligand of heme (shaded in light blue), and all cysteine residues were conserved in mature peroxidases 1–3 (Fig. 7). Concerning tyrosine residue, which is reported to be involved in binding of proteins to lignin and its related compound (25, 27), peroxidase 1 showed a higher tyrosine content (7 residues in deduced mature form) than peroxidase 2 or 3 (5 residues each). Among them, four tyrosine residues were conserved in peroxidases 1–3. Although neither peroxidase had repetitive proline-rich motifs found in extensins and PRPs, we found that only peroxidase 1 possesses three proline-rich regions. The sequences in the first and second proline-rich regions (Ser-Pro-Pro and Pro-Pro-Pro-Ser, respectively) were also found in the amino acid sequence predicted from the nucleotide sequence of tobacco extensin gene (28). Maize extensin gene was shown to encode repeats of a proline-rich motif consisting of 16 amino acids (29), partial sequence of which was identical with that (Pro-Pro-Thr-Pro) in the third proline-rich region. Surprisingly, the amino acid sequence (Val-Val-Pro-Met-Asp-Pro-Pro-Thr-Pro-Ala) of the third region were found to bear 80% identity and 90% homology to a region (Val-Val-Pro-Asp-Pro-Pro-Pro-Pro-Pro-Thr-Pro-Ala) of the structural proteins of the giant cockroach (Blaberus craniifer) (30). In contrast, mature peroxidases 2 and 3 did not possess any proline-rich domains. Thus, molecular characterization established that among the three isozymes, only peroxidase 1 has structural protein-like motifs as well as regions critical for peroxidase activity.

**DISCUSSION**

In present study, we have discovered that peroxidase 1 in S. baicalensis has quite novel properties. Elicitor treatment or mechanical damage of the callus led to a decrease in ionically bound wall peroxidase 1, and this decrease was confirmed by immunostaining to be due to its insolubilization in cell wall. Peroxidase 1 is the first enzyme insolubilized in response to
oxidative stress. Chemical and enzymatic treatments could not solubilize peroxidase 1 once it was insolubilized. These properties of peroxidase 1 are identical with those of extensins and PRPs in French bean and soybean cells (1). Because insolubilization of both structural proteins has been believed to strengthen the cell wall barrier against invading pathogen, peroxidase 1 may have a similar function. Plant peroxidases have been shown to have various enzymatic properties, but peroxidases having structural protein-like properties have not been reported.

The mechanism of peroxidase insolubilization is also similar to that of the cross-linking of extensins and PRPs. As soon as *Scutellaria* callus exhibits oxidative burst in response to elicitor treatment or mechanical damage, peroxidase 1 is oxidatively cross-linked to cell walls in the presence of the resulting H$_2$O$_2$. This reaction is catalyzed by peroxidase 1 itself. It is notable that enzyme acts as its substrate. On the other hand, extensins are shown to be polymerized by *in vitro* peroxidase reaction in the absence of cell wall (7), whereas polymerization of peroxidase 1 has not been observed under similar conditions. For the mechanism of polymerization of extensins, it is proposed that they are polymerized by linking each other through intermolecular tyrosine-tyrosine (dityrosine or isodityrosine) bonds (1, 31). Because the tyrosine content in peroxidase 1 is lower than that in extensins, intermolecular tyrosine-tyrosine linkages may be difficult to be formed in peroxidase 1 as compared with extensins.

We conclude that peroxidase 1 is cross-linked to the lignin moiety in cell wall, based on the facts that this enzyme covalently binds only to lignocellulose and that the cross-linked peroxidase 1 cannot be solubilized by treatment with cellulase or anhydrous hydrofluoric acid. Evans and Himmelsbach (17) reported that during *in vitro* synthesis of lignin, peroxidase reaction catalyzes formation of tyrosine-lignin bonds between the enzyme and the resulting lignin-like products. Therefore, it is likely that peroxidase 1 is also cross-linked to cell wall through tyrosine-lignin bond. Peroxidase bound to cell walls via tyrosine-lignin bond has been identified in flax plants (*Linum usitatissimum*) (18) and has been shown to be solubilized by limited proteolysis (25). We treated the cell wall fraction of the elicited *Scutellaria* callus under similar conditions, but cross-linked peroxidase 1 was not solubilized, suggesting that insolubilized peroxidase 1 may be more rigidly bound to cell wall than flax peroxidase or may resist hydrolysis by protease. Constitutive presence of covalently bound wall peroxidases has been confirmed in various plants, including flax plants (15–18), whereas the physiological roles of these enzymes and the mechanisms of covalent bond formation remain unclear.

Insolubilization of peroxidase 1 was initiated within 10 min and almost completed within 60 min. This response is apparently faster than transcription-dependent defense responses, such as phytoalexin accumulation, which are usually observed at more than 2 h after elicitation (32). Accordingly, insolubilization of peroxidase 1 can be regard as one of the earlier defense responses. Previously we demonstrated preexistence of large amounts of peroxidases 1 and 2 in the cell walls of *S. baicalensis*, and this constitutive expression is considered to enable the rapid response. Interestingly, the activities of ionically bound wall peroxidases were also shown to continue to decrease until 60 min after treatment of *Scutellaria* cells with elicitor (19). This rapid decrease may be due to cross-linking of NaCl-soluble peroxidase 1. Insolubilization of extensins and
PRPs is somewhat more quickly initiated and completed, compared with peroxidase 1. *Scutellaria* cells immediately metabolize large amounts of H$_2$O$_2$ in a response to elicitor treatment, but addition of excess H$_2$O$_2$ only slightly enhanced its insolubilization. Therefore, scarcity of H$_2$O$_2$ is not the cause of the slower insolubilization rate of peroxidase 1. The lower tyrosine content in peroxidase 1 may account for its slower insolubilization as compared with extensins, because McDougall et al. (27) demonstrated that the presence of tyrosine residues accelerates the cross-linking of synthetic proteins to lignin-like products.

In the present study, we have identified a new isozyme (peroxidase 3), together with peroxidases 1 and 2, in *S. baicalensis*, whereas peroxidases 2 and 3 are not insolubilized by oxidative stress. Because we assumed that the divergent properties between these peroxidases are due to their structures, we examined their amino acid sequences. Consequently, we confirm that the primary structures of peroxidases 2 and 3 are similar to each other (59% identity) but show lower homology to confirm that the primary structures of peroxidases 2 and 3 are oxidative stress. Because we assumed that the divergent properties of peroxidase 1 are identical with those in peroxidases 2 and 3. A tyrosine residue is presumed to contribute to binding of peroxidase and synthetic products. Whereas peroxidase 1 contains more of both tyrosine and proline residues than peroxidases 2 and 3. A tyrosine residue is assumed to contribute to binding of peroxidase and synthetic proteins to lignin (17, 25, 27), and the locations of the four tyrosine residues (Tyr', Tyr$^{184}$, Tyr$^{233}$, and Tyr$^{234}$) in peroxidase 1 are identical with those in peroxidases 2 and 3. Because peroxidases 2 and 3 are not insolubilized, the other tyrosine residues (Tyr$^{184}$, Tyr$^{117}$, and Tyr$^{200}$) seem more significant for insolubilization of peroxidase 1 than the conserved ones. These three residues are located between the first and third proline-rich regions, whereas peroxidases 2 and 3 contain no tyrosine residue except for the conserved residue (Tyr$^{184}$) in the corresponding regions. Hence, insolubilization of *Scutellaria* peroxidase may depend on the number or appropriate location of tyrosine residues in the middle domain containing approximately 100 amino acid residues. Moreover, the novel features are found in the locations of proline residues in peroxidase 1. Peroxidase 1 possesses three proline-rich regions in its molecule, and these amino acid sequences are also found in those predicted from genes encoding tobacco extensin (28), maize extensin (29), and a giant cockroach structural protein (30), assuming that this isozyme can act as a structural protein. In contrast, peroxidases 2 and 3 have no significant characteristics except for regions with high homology to other plant peroxidases. We examined proline-rich regions in peroxidases from other plants using SwissProt, whereas no proline-rich regions were found in 10 peroxidases with relatively high homology to peroxidase 1, except for rice peroxidase, having only one region (Pro-Pro-Pro).

Thus, we confirm that peroxidase 1 consists of the domains critical for peroxidase activity, the motifs characteristic of structural proteins, and the tyrosine residues presumably involved in cross-linking to cell wall. The properties and molecular characteristics of peroxidase 1 support the notion that like extensins and PRPs, this enzyme also play potentially important roles in stiffening of cell walls in *S. baicalensis*. Previously, we demonstrated that peroxidase 1 effectively metabolizes large amounts of H$_2$O$_2$ in the *Scutellaria* cells. Accordingly, peroxidase 1 is a quite versatile enzyme involved in various defense reactions.

REFERENCES

1. Bradley, D. J., Kjellbom, P., and Lamb, C. J. (1992) Cell 70, 21–30
2. Varner, J. E., and Lin, L.-S. (1989) Cell 56, 231–239
3. Datta, R., Schmidt, A., and Marcus, A. (1989) Plant Cell 1, 945–952
4. Hong, J. C., Nagao, T. R., and Key, J. L. (1990) J. Biol. Chem. 265, 2470–2475
5. Cooper, J. B., and Varner, J. E. (1984) Plant Physiol. 76, 414–417
6. Everdeen, D. S., Kiefer, S., Willard, J. J., Muldoon, E. P., Dey, P. M., Li, X.-B., and Lampert, D. T. A. (1988) Plant Physiol. 87, 616–621
7. Brownleader, M. D., Ahmed, N., Trean, M., Chaplin, M. F., and Dey, P. M. (1995) Plant Physiol. 109, 1115–1123
8. Lagrimini, L. M., Burkhart, W., Moyer, M., and Rothstein, S. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7542–7546
9. Asada, K. (1992) Physiol. Plant. 85, 235–241
10. Hinnman, R. L., and Lang, J. (1965) Biochemistry 4, 448–452
11. Grisebach, H. (1981) in The Biochemistry of Plants (Conn, E. E., ed) Vol. 7, pp. 475–478, Academic Press, New York
12. Espelie, K. E., Franceschi, V. R., and Kolattukudy, P. E. (1986) Plant Physiol. 81, 487–492
13. Christensen, J. H., Baun, G., Weidner, K. G. Montague, M. V., and Boerjan, W. (1998) Plant Physiol. 116, 125–135
14. Foyer, C. H., and Halliwell, B. (1976) Planta 13, 21–25
15. Ridge, I., and Osborne, D. J. (1971) Nat. New Biol. 229, 205–208
16. Goldberg, T., Imberty, A., Liberman, M., and Prat, R. (1986) in Molecular and Physiological Aspects of Plant Peroxidases (Greppin, H., Penel, C., and Gaspar, T. ed) pp. 208–220, University of Geneva, Geneva
17. Evans, J. J., and Himmelbach, D. S. (1991) J. Agric. Food. Chem. 39, 830–832
18. McDougall, G. J. (1992) Phytochemistry 31, 3385–3389
19. Morimoto, S., Tateishi, N., Matsuda, T., Tanaka, H., Taura, F., Furuya, N., Matsuyma, N., and Shoyama, Y. (1998) J. Biol. Chem. 273, 12606–12611
20. Morimoto, S., Harita, T., and Shoyama, Y. (1995) Planta 195, 535–540
21. Mustonen, T., and Skoog, P. (1982) Physiol. Plant. 54, 473–497
22. Mobassaleh, M., Mishra, K., and Keusch, G. T. (1995) J. Clin. Invest. 95, 295–300
23. Srisuma, N., Ruengsakulrach, S., Ubersax, M. A., Bennink, M. R., and Hammerschmidt, R. (1991) J. Agric. Food. Chem. 39, 855–858
24. O’Neill, M. A., and Selvendran, R. R. (1980) Biochem. J. 177, 53–63
25. McDougall, G. J. (1995) Phytochemistry 33, 765–767
26. Kieliszewski, M., and Lampert, D. T. A. (1991) Plant Physiol. 95, 823–827
27. McDougall, G. J., Stewart, D., and Morrison, I. M. (1996) Phytochemistry 41, 43–47
28. Keller, H., and Lamb, C. J. (1989) Genes Dev. 3, 1639–1646
29. Kieliszewski, M., Leykam, J. F., and Lampert, D. T. A. (1990) Plant Physiol. 92, 316–326
30. Jensen, U. G., Rothmann, A., Skou, L., Andersen, S. O., Roepstorff, P., and Hejrup, P. (1997) Insect Biochem. Mol. Biol. 27, 109–120
31. Fry, S. C. (1982) Biochem. J. 204, 449–455
32. Dixon, R. A., and Harrison, M. J. (1990) Adv. Genet. 28, 165–234