Pergamon

0031-9422(94)00665-2

*Phytochemistry*, Vol. 39, No. 1, pp. 29–32, 1995

0031-9422/95 $9.50 + 0.00

# TWO CATIONIC PEROXIDASES FROM CELL WALLS OF *ARAUCARIA ARAUCANA* SEEDS

ALEJANDRO RIQUELME* and LILIANA CARDEMIL†

Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

(*Received in revised form* 28 *July* 1994)

**Key Word Index**—*Araucaria araucana*; Araucariaceae; seeds; basic peroxidases; cell wall protein; homology.

**Abstract**—We have previously reported the purification and partial characterization of two cationic peroxidases from the cell walls of seeds and seedlings of the South American conifer, *Araucaria araucana*. In this work, we have studied the amino acid composition and $NH_2$-terminal sequences of both enzymes. We also compare the data obtained from these analyses with those reported for other plant peroxidases. The two peroxidases are similar in their amino acid compositions. Both are particularly rich in glycine, which comprises more than 30% of the amino acid residues. The content of serine is also high, *ca* 17%. The two enzymes are different in their content of arginine, alanine, valine, phenylalanine and threonine. Both peroxidases have identical $NH_2$-terminal sequences, indicating that the two proteins are genetically related and probably are isoforms of the same kind of peroxidase. The amino acid composition and $NH_2$-terminal sequence analyses showed marked differences from the cationic peroxidases from turnip and horseradish.

## INTRODUCTION

Plant peroxidases (EC 1.11.1.7, donor: hydrogen peroxide oxidoreductase) are glycoproteins that contain protohaem IX. They are widely distributed in plant tissues and are particularly abundant in cell walls [1, 2], where they are probably involved in cell wall metabolism. Some of them appear to be isoforms of one class of peroxidase, although it is not clear to what extent these are different gene products or are the results of post-transitional modifications of one or few gene products [3].

Particularly well-studied are the major cationic peroxidases isolated from turnip and horseradish [4–8]. The complete amino acid sequences of turnip 7 (TP 7) and horseradish (HRP) peroxidases are known [9]. In the case of HRP, four genes for related peroxidases have been isolated [10, 11].

Peroxidases have been implicated in a variety of physiological processes. For example, they participate in the biosynthesis and degradation of lignin [12, 13], in the repairing response to wounding [14], in the biosynthesis and polymerization of extensin [15] and in auxin metabolism [16, 17].

We have previously reported the purification and characterization of two peroxidases from the cell walls of seeds and seedlings of *Araucaria araucana* [18]. These are cationic peroxidases with a p*I* of 10.5, optimal pH of 5, a buoyant density of 1.333 g ml$^{-1}$ and a $M_r$ of 83 000 and 145 000. The peroxidases have a $K_m$ app of 13.6 mM for $H_2O_2$ and 3.4 mM for phenylenediamine (O-PDA). The $V_{max}$ is 525 $\mu$mol O-PDA oxidized min$^{-1}$ mg$^{-1}$. The enzymes are glycosylated and the carbohydrate portion linked to the proteins represents 10–12%, with galactose comprising more than 60% of the sugar residues.

The purpose of the present work was to study the amino acid composition and the $NH_2$-terminal sequence of these hemoproteins in order to determine if they are genetically related and therefore could be defined as different isoenzymes of the same class of peroxidase.

## RESULTS AND DISCUSSION

### *Amino acid composition*

Table 1 shows the relative content of amino acid residues of each peroxidase. The number of residues for each amino acid was obtained from the mass of the protein portion of the molecule. These were estimated from the $M_r$ of the peroxidases from which the carbohydrate portions were subtracted. The results obtained demonstrated that the two peroxidases are very similar in their amino acid composition. Both are particularly rich in glycine (32.81 and 36.16%) followed in decreasing order by serine (15.65 and 18.84%) and alanine (10.23 and 6.86%). The peroxidases differ, however, in the amount of valine and phenylalanine, which were present only in the

*Present address: Departmento de producción Agrícola, Facultad de Ciencias Agrarias Y Forestales, Univesidad de Chile, Santa Rosa 11315, Santiago, Chile.

†Author to whom correspondence should be addressed.

Table 1. Amino acid composition of *Araucaria araucana* cell wall peroxidases. The composition is compared with those of HRP and TP7 peroxidases

| | *Araucaria araucana* | | | |
|---|---|---|---|---|
| | $M_r$ 83000 | $M_r$ 145000 | HRP* | TP7† |
| | % mol | | | |
| Lys | 5.37 (37) | 4.92 (66) | 1.98 (6) | 3.38 (10) |
| His | 2.93 (20) | 1.73 (23) | 1.32 (4) | 1.01 (3) |
| Arg | 2.26 (16) | 4.12 (55) | 7.92 (24) | 5.74 (17) |
| Asx | 4.87 (34) | 4.52 (61) | 12.54 (38) | 13.18 (39) |
| Glx | 8.60 (59) | 7.03 (94) | 6.93 (21) | 4.73 (14) |
| Gly | 32.81 (226) | 36.16 (484) | 10.23 (31) | 8.11 (24) |
| Ala | 10.23 (71) | 6.86 (92) | 7.59 (23) | 10.81 (32) |
| Val | 3.97 (27) | 0.00 (0) | 9.24 (28) | 6.42 (19) |
| Leu | 3.19 (22) | 3.74 (50) | 7.92 (24) | 7.09 (21) |
| Ile | 1.90 (13) | 1.76 (24) | 4.95 (15) | 5.07 (15) |
| Pro | 4.92 (34) | 5.87 (79) | 4.95 (15) | 3.72 (11) |
| Ser | 15.65 (108) | 18.84 (252) | 7.59 (23) | 14.19 (42) |
| Thr | 2.50 (17) | 4.32 (58) | 7.59 (23) | 5.41 (16) |
| Cys | N.D. | N.D. | 2.64 (8) | 2.70 (8) |
| Met | N.D. | N.D. | 1.32 (4) | 2.03 (6) |
| Phe | 0.75 (5) | 0.00 (0) | 4.29 (13) | 4.73 (14) |
| Tyr | 0.00 (0) | 0.00 (0) | 0.66 (2) | 1.35 (4) |
| Trp | N.D. | N.D. | 0.33 (1) | 0.34 (1) |
| Total | 100 (689) | 100 (1338) | 100 (303) | 100 (296) |

*Data from ref. [19].
†Data from ref. [9].
Numbers in parentheses are numbers of residues per molecule.
N.D., Not determined.

peroxidase of $M_r$ 83000. This, on the other hand, had significantly less threonine and arginine than the peroxidase of $M_r$ 145000. The basic p*I* of these two peroxidases indicates that most of the Glx and Asx residues are present as glutamine and aspargine in the native proteins.

Table 1 also compares the amino acid composition of *A. araucana* peroxidases with the amino acid content of the cationic peroxidases present in horseradish and in turnip [9, 19]. These two peroxidases were markedly different from those in *A. araucana*. They have considerably less glycine and serine and more arginine, aspartic acid/aspargine, valine, leucine and isoleucine, although there are some similarities in the mol % of alanine, proline and histidine.

It should be pointed out that the high glycine content of the two *A. araucana* peroxidases is not an artifact derived from the tricine buffer and/or from the preparative purification of the proteins by blotting on to Immobilon Transfer membranes. Control samples prepared with a protein-free piece of the same membrane used in the electroblotting and, therefore, run under the same conditions as the pieces of membrane containing the proteins, did not release glycine after hydrolysis with 6 N HCl.

Glycine-rich proteins (GRPs) are present in plant cell walls [20–22]. The function of the GRPs is unknown; however, they have been reported to be structural proteins with a $\beta$-pleated sheet configuration, localized in the protoxylem elements and clearly associated with cell walls that are going to be lignified [23, 24]. The *A. araucana* cell wall proteins are, therefore, the first reported GRPs with peroxidase activity.

### $NH_2$-*Terminal sequence*

A sequence of 20 amino acids out of a total 689 residues was obtained for the $M_r$ 83000 peroxidase, while a sequence of 15 amino acids out of a total 1338 residues was obtained for the $M_r$ 145000 peroxidase (Table 2). In both peroxidases, the sequences are identical for the first 15 amino acids. However, these two sequences are very different from the $NH_2$-terminal sequences of HRP and TP 7 [9, 19]. Only the threonine in position 3 and the serine in position 10 are found in the four sequences shown in Table 2. The proline of position 4 found in the *A. araucana* sequences is also conserved in the same position in HRP. The dissimilarity found between the $NH_2$-terminal sequence of *A. araucana* and other plant peroxidases may be the consequence of the phylogenetic distance between conifers and angiosperms. Moreover, the sequence reported here has been found to be unique to *A. araucana* and, therefore, different from all other peroxidases reported. Indeed, a homology search in the National Biomedical Research Foundation data bank of Monsanto Corporate Research Laboratory, in the Gene Bank and in the European Molecular Biology Laboratory data bank, demonstrated that the *A. araucana* $NH_2$-terminal sequence does not have homology with any other protein sequence reported so far. However, it is important to note that there is not much information available on $NH_2$-terminal sequences of conifer proteins.

A characteristic feature of all peroxidases is the occurrence of at least two histidine residues present in highly conserved regions, one in the heme binding site and another in the catalytic site [7, 9, 10]. In *A. araucana* peroxidases, there is a relatively high number of histidine residues. However, only further analyses will determine whether or not the two conserved histidine residues are also present in *A. araucana* peroxidases. Probably, conserved peroxidase regions may be present in these *A. araucana* cell wall proteins, in spite of the large differences in the amino acid composition and the $NH_2$-terminal sequences found between other peroxidases. However, it is important to point out that the sequences reported here comprise 15 and 20 amino acids of the $NH_2$-terminal end of proteins which have a total of 689 for the $M_r$ 83000

Table 2. $NH_2$-terminal sequences of *Araucaria araucana* peroxidases. The amino acid residues are represented in the single-letter code according to IUPAC-IUB nomenclature

| | |
|---|---|
| $M_r$ 83000 | (A) M T P E Q V Q S S L E P Y I S Y L T A |
| $M_r$ 145000 | (A) M T P E Q V Q S S L E P Y I |
| HRP | Z L T P T F Y D N S C P N V S N I V R D |
| TP 7 | Z L T T N F Y S T S C P N L L S T V K S |

HRP and TP7 $NH_2$-terminal sequences are from refs [9] and [19].

peroxidase and 1338 for the $M_r$ 145000 peroxidase. In any case, the differences found between TP 7 and HRP, and those from *A. araucana* peroxidases are not surprising, because large differences in the molecular structure have also been found among plant peroxidases. Indeed, between TP7 and HRP there is only 49% homology [9].

## EXPERIMENTAL

*Plant material.* Seeds of *A. araucana* (Mol.) K. Koch, sepd from seed coats, were sterilized with 5% commercial bleach for 15 min and rinsed several times with $H_2O$. Seeds were germinated on wet vermiculite in growth chambers at 20° for 24 hr.

*Cell wall preparation.* Cell walls were purified and extracted for proteins with 0.2 M $CaCl_2$ in 2 mM $Na_2S_2O_5$ according to the method described previously [18, 25]. After extraction, proteins were pptd with 5 vols of cold $Me_2CO$. The protein pellet was sepd by centrifugation at 15000 *g* and resuspended in 0.1 M Tris–HCl, pH 6.8. Proteins were determined by the method of ref. [26] using BSA as standard.

*Amino acid analysis.* Amino acid compositions were determined after prep. purification of each protein. For this, cell wall peroxidases were sepd by SDS–PAGE at the same concn described previously [18, 25], loading 100 μg of protein in each well of the gel. The running buffer was 0.192 M tricine in 0.025 M Tris containing 0.1% SDS. After electrophoresis, the proteins were electroblotted overnight at 150 mA at 4° on to polyvinylidene difluoride (PVDF) membranes, also called Immobilon Transfer membranes, according to the procedure of ref. [27]. The transfer buffer was 10 mM 3-(cyclohexylamino)-1-propane-sulphonic acid (CAPS), pH 13. After transfer, the membrane was washed thoroughly in distilled $H_2O$ and the proteins visualized on the Immobilon Transfer membrane with Coomassie Blue. The piece of membrane containing each protein was cut out and the protein hydrolysed on the membrane at 110° in 6 N HCl. A protein-free piece of the same transfer membrane which received the proteins during electroblotting was run as control and hydrolysed under the same conditions. The times of hydrolysis were 24 and 48 hr, running each hydrolysis in duplicate. After hydrolysis, the HCl was evapd. The released amino acids were extracted from the membranes with 500 μl of 70% EtOH. After extraction, the EtOH soln was lyophilized. The dried powder was resuspended in 90 μl 0.02 M Na citrate, pH 5.4. The soln containing the amino acids (50 μl) was injected into an amino acid analyser (Beckman 6300) and quantified by the colorimetric determination of the ninhydrin reaction.

*$NH_2$-Terminal sequence analysis.* Proteins were sepd by cationic neutral gel electrophoresis as described previously [18, 25]. After electrophoresis, the proteins were electroblotted overnight on to a PVDF membrane at 165 mA at 4° according to ref. [27]. The protein bands were visualized on the membrane with Coomassie Blue and the area of the membrane containing the protein was cut off in ribbons of 4 × 9 mm, each ribbon containing at least 20 μg of protein. The $NH_2$-terminal sequence was determined by Edman degradation using an automatic sequencer. The phenylthiohydantoin-amino acid derivatives (PTH-amino acid) were identified by reverse-phase HPLC analysis.

*Acknowledgements*—We thank Dr Joseph E. Varner, Department of Biology, Washington University, St. Louis, Missouri for advice and encouragement during development of this research and Angelica Vega for technical assistance. This research was supported by grant 7068, Program in Science and Technology Cooperation, Office of Research, U.S. Agency for International Cooperation, and DTI B 2806-8923 from Universidad de Chile.

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