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Research article

Purification and kinetic characterization of two peroxidases of *Selaginella martensii* Spring. involved in lignification

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ABSTRACT

Two cationic peroxidases from *Selaginella martensii* Spring. (SmaPrx2 and SmaPrx3) were purified using a three-step protocol which includes ammonium sulfate precipitation, adsorption chromatography on phenyl sepharose and cationic exchange chromatography on SP sepharose. The molecular mass for SmaPrx2 and SmaPrx3 was calculated to be 36.3 kDa and 45.6 kDa, respectively, according to MALDI-TOF/TOF. The isoelectric points were estimated in 9.2 and 9.5 for SmaPrx2 and SmaPrx3, respectively, according to isoelectrofocusing. Both enzymes show a typical peroxidase UV–visible spectrum with a Soret peak at 403 nm for SmaPrx2 and 404 nm for SmaPrx3. The specific activities showed against several substrates and the kinetic parameters suggest SmaPrx2 and SmaPrx3 have specific roles in cell wall formation and especially in lignin biosynthesis. Several peptides from tryptic digestion of both peroxidases were identified through MALDI-TOF MS/MS. The presence in these peptides of structural determinants typical of syringyl peroxidases indicates these proteins show no structural restrictions to oxidize syringyl moieties. These data, along with the *in vitro* capacity of using sinapyl alcohol as substrate and the low K_m in the μM range suggest these two peroxidases may be responsible for the oxidation of syringyl monolignols that leads to syringyl lignins biosynthesis.

1. Introduction

Classical secretory plant peroxidases (class III Prx, EC 1.11.1.7) are heme-containing glycoproteins able to oxidize different substrates using hydrogen peroxide as electron donor. Peroxidases can be divided into three structural classes: class I includes ascorbate peroxidase from higher plants and peroxidases from yeast and bacteria. Class II is found in fungi and composed of manganese peroxidases. Class III comprises classical secretory plant peroxidases [1].

Peroxidases have been implicated in several physiological roles, from germination to senescence. Some isoenzymes are constitutively expressed, as they play an important role in plant structure and development, while other peroxidase isoenzymes are induced in response to various stresses. Peroxidases are known to participate in phenolic compound oxidation [2], cell elongation [3], metabolism of both reactive oxygen species and reactive nitrogen species [4], phytohormone metabolism [5], cross-linking of cell walls components and suberin and lignin formation [6]. The specific functions of individual peroxidases are often difficult to assign because of their low substrate specificity and the presence of many isoenzymes [1]. In higher plants, the number of isoenzymes is extremely high, 73 genes correspond with class III peroxidases within the Arabidopsis genome [7] and 138 in rice [8]. Several other isoforms can be generated by post-transcriptional and post-translational modifications. All these features entail that a complete understanding of the role of peroxidases can only be accomplished through a detailed analysis of spatial and temporary expression of each peroxidase isoenzyme, as well as its substrate specificity [9].

Despite the redundancy and the lack of substrate specificity, different responses to several stresses or growth conditions have been found in rice and Arabidopsis, indicating diverse roles for the different isoforms [5,7,8]. Moreover, several works report the direct implication of peroxidase isoenzymes in lignification [10,11].

Lignins are three-dimensional heteropolymers resulting from the oxidative coupling of three *p*-hydroxycinnamyl alcohols (monolignols): *p*-coumaryl, coniferyl and sinapyl alcohols [12]. The cross-coupling reaction of monolignol radicals produces a hydrophobic heteropolymer composed by *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively. The last step of lignin

Abbreviations: CAPS, 3-[cyclohexylamino]-1-propane-sulfonic acid; G, guaiacyl; H, *p*-hydroxyphenyl; IAA, indole-3-acetic acid; IEF, isoelectrofocusing; MALDI-TOF, matrix assisted laser desorption ionization–time-of-flight; S, syringyl; SmaPrx, peroxidase purified from *Selaginella martensii*; TMB, 3,5,3′,5′-tetramethylbenzidine.

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biosynthesis is the oxidation of monolignols, which is driven by laccases [13] and mainly peroxidases [14]. However, peroxidases are able to oxidize monolignols to produce H, G and S units whereas laccases only generate G units [13].

According to their isoelectric point, peroxidases can be classified into acidic, neutral and basic. It has been reported that both anionic and cationic peroxidases play a role in lignin formation [14]. El-Mansouri et al. [15] overexpressed a basic peroxidase from tomato resulting in an increment of lignin content in transgenic plants. In transgenic lines of aspen, lignin content was reduced up to 20% with the down-regulation of PrxA3a, an acidic peroxidase [10]. Nevertheless, the down-regulation of an acidic isoenzyme of tobacco did not produce a diminution in lignification [16]. These results suggest it is not an individual peroxidase the only responsible for lignification, but some isoenzymes participate in lignin formation. The difference between acidic and cationic peroxidases may not be the involvement in lignification but the differential participation in lignin formation. Thus, antisense suppression of basic peroxidases leads to a decrease in both G and S units in tobacco [17], but antisense suppression of acidic peroxidases from poplar only drives a reduction of G units levels [10].

One of the pivotal points in plant evolution is the lignification, which prevents plants from desiccation and allowed the land colonization. Vascular tissues are impregnated with lignins, which provide mechanical strength and make cell walls water impermeable, allowing the transport of solutes through the xylem. Vascular plants appeared 410 Myr ago, then diverged into several lineages, among them, the only surviving: euphyllophytes (ferns and seed plants) and lycophytes. Regarding lignin composition, angiosperms are usually formed of G and S units, while in gymnosperms lignins lack S units, and are mainly composed of G units, with a minor proportion of H units, though there are some exceptions [18]. Ferns usually show the same pattern as gymnosperms, although the presence of S units has been previously described [19,20], and even in non vascular plants the presence of S units has been previously reported [20].

Selaginella martensii is a lycophyte, belonging to class Lycopsida, order Selaginellales. Lycophytes, also called spikemosses, constitute a monophyletic group with origins dating to the late Silurian/early Devonian (400 Myr ago). Selaginella is considered a key point in studying evolution, as it preserves some features typical of non vascular plants but it also presents some evolutionary innovations, such as vascular tissues, leaves, stems and lignification. One member of Selaginella, *Selaginella moellendorffii*, has been fully sequenced and has a genome size of only ~100 Mbp, which is the smallest genome size of any plant reported [21]. This species is used as a new model to study the pathway of lignin biosynthesis, as it presents two novel enzymes that divert the metabolic flux from guaiacyl units to syringyl lignins and are reported to have independent origins from angiosperms via convergent evolution. These two proteins (ferulic acid 5-hydroxylase, F5H and caffeic acid/5-hydroxyferulic acid *O*-methyltransferase, COMT) are functionally analogous to angiosperm enzymes but are not structurally related [22,23]. Regarding *S. martensii*, it has been previously reported the capacity of its peroxidases to oxidize sinapyl alcohol and sinapyl aldehyde [20], which is in good agreement with the angiosperm-like percentage of S lignins (70%) found in the lycophyte [19,20]. However, the isoenzyme responsible for catalyzing the oxidation of sinapyl alcohol remains unknown.

In this paper we report the presence of several basic peroxidases in a protein extract of *S. martensii*. Two isoforms were purified and further characterized with biochemical and proteomic methods. These peroxidases SmaPrx2 and SmaPrx3 may have functions in cell wall formation and more specifically in syringyl lignins formation as shown by the capacity of oxidizing sinapyl alcohol.

2. Results

2.1. Peroxidase and lignin localization

S. martensii has been reported to account for syringyl moieties in its lignins [19]. Thioacidolysis of the lignifying cell walls of *S. martensii* fronds revealed the presence of thioethylated monomers (*erythro* and *threo* isomers) arising from the aryl-glycerol-β-aryl ether (β-*O*-4) structures derived from sinapyl alcohol (Fig. 1A, B). To study the specific sites of S lignins deposition, we performed the Maüle test, which is specific for syringyl groups [22]. The results (Fig. 1C) showed the presence of lignins in both xylem and epidermis but S lignins were deposited only in the epidermal and sub-epidermal/cortical tissues, as shown by the red staining. On the other hand, the brown staining in the xylem indicates it is mainly composed of G units. Both epidermis and xylem were also green-stained in the presence of TMB (Fig. 1D), which reveals the peroxidase enzymatic activity. This colocalization of peroxidase and lignins has been widely described [19,20] suggesting the involvement of peroxidase isoenzymes in lignin formation.

Cationic peroxidases have been hypothesized to be responsible for sinapyl alcohol oxidation in several works [24–26]. Thus, we investigated the peroxidase isoenzyme pattern from a total protein extract of *S. martensii* fronds by means of isoelectrofocusing analysis. The results show the presence of acidic, neutral and basic peroxidases (Fig. 1E). Among the latter, there are several isoenzymes with a strongly basic pI, which may be candidates to catalyze sinapyl alcohol oxidation. In a previous work, it has been reported that a total protein extract of *S. martensii* fronds contained peroxidases able to oxidize both sinapyl alcohol and aldehyde [20]. For this reason, we considered a purification of several basic peroxidases that may be able to oxidize sinapyl alcohol and therefore, candidates to fulfill the last step of S lignins biosynthesis.

2.2. Purification of SmaPrx2 and SmaPrx3

To perform peroxidase purification, we followed a three-step protocol including ammonium sulfate precipitation, adsorption chromatography on phenyl sepharose and cationic chromatography on SP sepharose. The first step was a protein precipitation with ammonium sulfate. Previously, we performed an ammonium sulfate fractionation to determine, according to protein and peroxidase patterns, which fractions contained the proteins of interest. Six fractions were chosen to be examined: 0–25; 25–40; 40–50; 50–65; 65–80 and 80–95% $(NH_4)_2SO_4$. After electrophoretic analysis, the fractions from 0 to 80% $(NH_4)_2SO_4$ showed similar protein and peroxidase patterns and therefore, we decided to join them as a single fraction in the following ammonium sulfate precipitations. The fraction from 80 to 95% $(NH_4)_2SO_4$ resulted in a marked reduction of protein quantity, with a much simpler pattern than 0–80% $(NH_4)_2SO_4$ fraction (data not shown). Hence, we considered two protein fractions, the first one from 0 to 80% of ammonium sulfate and the second one, from 80 to 95% of ammonium sulfate. Each fraction was pooled into a phenyl sepharose chromatography where the 0–80% ammonium sulfate fraction was resolved into two major fractions of peroxidases (F1 and F2, Fig. 2A). The F2 was then loaded into a cationic exchange chromatography, and the peroxidase bound to SP sepharose matrix was eluted with a linear gradient of 0–1 M KCl. The enzyme was eluted at a salt concentration of 0.15 M (Fig. 2B). After this step, the specific activity reached 61300 nkat mg^{-1} protein (Table 1).

The 80–95% ammonium sulfate fraction was pooled into a phenyl sepharose chromatography, where only a peroxidase peak was eluted (Fig. 3A). These fractions were concentrated, dialyzed and loaded into a cationic exchange chromatography. Considering