Purification, crystallization and preliminary crystallographic analysis of peroxidase from the palm tree Chamaerops excelsa
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Plant peroxidases are presently used extensively in a wide range of biotechnological applications owing to their high environmental and thermal stability. As part of efforts towards the discovery of appealing new biotechnological enzymes, the peroxidase from leaves of the palm tree *Chamaerops excelsa* (CEP) was extracted, purified and crystallized in its native form. An X-ray diffraction data set was collected at a synchrotron source and data analysis showed that the CEP crystals belonged to the orthorhombic space group P2₁2₁2₁, with unit-cell parameters \( a = 70.2, b = 100.7, c = 132.3 \) Å.

1. Introduction

Enzymes that can tolerate extreme conditions of pH and temperature are widely used in biotechnological applications, particularly in the biocatalytic industry. However, the design and development of extremoenzymes are limited since the molecular basis of their conformational stability is still not completely understood. In general, peroxidases are known to be environmentally stable enzymes, especially those derived from thermophilic microorganisms (Loprasert *et al.*, 1988; Rabe *et al.*, 2008; Gudelj *et al.*, 2001; Kengen *et al.*, 2001; Apitz & van Pée, 2001) and superior plants (Mchedlishvili *et al.*, 2005; McEldoon & Dordick, 1996; Rani & Abraham, 2006). Peroxidases are involved in several physiological processes in plants, including growth regulation (Camp, 1991), lignification and cell-wall formation (Wallace & Fry, 1999), protection against pathogens and wound healing (McLusky *et al.*, 1999) and abiotic and biotic stress responses (Bolwell *et al.*, 2002; Avsian-Kretchmer *et al.*, 2004). They are haem-containing oxidoreductases that make use of hydrogen peroxide to oxidize several organic and inorganic substrates (Dawson, 1988). Based on structural properties (Welinder *et al.*, 1992), the plant peroxidase family is classified into class III (EC 1.11.1.7), which consists of secretory enzymes containing one N-terminal peptide signal, two conserved calcium ions and four disulfide bridges. These enzymes have conserved arginine and histidine residues adjacent to the haem-binding site and extra helices that play a role in the accessibility of the substrate to the haem edge (Gajhede *et al.*, 1997). They are also glycoenzymes bearing between two and eight asparagine-linked glycans which can contribute positively to protein stabilization (Wang *et al.*, 1996).

Horseradish peroxidase (HRP) is the most extensively studied peroxidase and is commercially used in biosensors and conjugates in immunoassays (Azevedo *et al.*, 2003). Soybean seed coat peroxidase (SBP) was isolated and characterized by McEldoon & Dordick (1996) and has also been considered for use in biotechnological applications owing to its higher thermostability compared with HPR (Ryan *et al.*, 2006). Towards the identification of further commercially promising stable and active peroxidases, the molecular and catalytic properties of several other plant peroxidases from fruit (Dubey *et al.*, 2007), tea leaves (Kvaratskhelia *et al.*, 1997), castor bean (Kumar *et al.*, 2008) and African and royal palm trees (Rodríguez *et al.*, 2002; Zamorano *et al.*, 2008; Watanabe *et al.*, 2010) have been studied. Moreover, crystallographic structures of class III plant peroxidases from
horseradish (Gajhede et al., 1997), peanut (Schuller et al., 1996), barley (Henriksen et al., 1998), soybean (Henriksen et al., 2001) and the royal palm tree Royostonea regia (Watanabe et al., 2010) have also been elucidated. In this work, we describe the isolation, purification and crystallization of the Chamaerops excelsa palm-tree peroxidase (CEP) and its preliminary X-ray diffraction analysis.

2. Experimental methods

2.1. Protein isolation and purification

CEP was purified from the palm tree C. excelsa as described previously (Watanabe et al., 2007; Zamorano et al., 2009) but with essential modifications. Leaves (1820 g) from a three-year-old palm tree were milled and homogenized in 7.28 l distilled water for 22–24 h at room temperature. Excess material was removed by vacuum filtration and centrifugation (10 000g and 277 K for 15 min). Pigments were extracted by phase separation over 20–22 h at 277 K after the addition to the supernatant of solid PEG to 14%(w/v) and solid ammonium sulfate to 10%(w/v). Two phases were formed after the addition of ammonium sulfate: an upper polymer phase (dark brown in colour) that contained pigments, phenols, polyphenols, oxidized phenols and PEG and a lower aqueous phase (yellow in colour) containing peroxidase. Each phase consisted of 50% of the initial volume. The phases were separated and the phase containing peroxidase activity was centrifuged. The clear supernatant containing peroxidase activity was titrated with ammonium sulfate to a conductivity value of 232 mS cm⁻¹ and applied onto a Phenyl-Sepharose column (1.5 × 35 cm) equilibrated with 100 mM phosphate buffer pH 6.5 with 1.7 M ammonium sulfate, which has the same conductivity as the sample. The enzyme was eluted with 100 mM phosphate buffer pH 6.5 plus 0.2 M ammonium sulfate at a flow rate of 1 ml min⁻¹. 15 ml fractions were collected and those showing peroxidase activity were dialyzed against 5 mM Tris buffer pH 9.3 for 72 h with constant stirring at 277–278 K. These fractions were membrane-concentrated (Amicon, 10 kDa cutoff) to 15 ml and applied onto a TSK-Gel DEAE-5PW column (1.5 × 30 cm) equilibrated with 5 mM Tris buffer pH 9.3. Elution was carried out with a linear 0–300 mM NaCl gradient in the same buffer at a flow rate of 1 ml min⁻¹. The fractions with peroxidase activity were collected, membrane-concentrated (Amicon, 10 kDa cutoff) and applied onto a Superdex 200 column equilibrated with 5 mM Tris buffer pH 9.3. Elution was carried out using the same buffer at a flow rate of 1 ml min⁻¹. Finally, the peroxidase was dialyzed against distilled water and freeze-dried.

Protein purity and quality were analyzed by native and denaturing polyacrylamide gel electrophoresis (PAGE) using gel concentrations of 8–25% gradient and 15% SDS, respectively, as well as by UV-visible spectrophotometry (RZ = A₂₈₀/A₂₆₀ = 2.8–3.0). Analysis of the oligomeric state and polydispersity of the enzyme at three different concentrations was carried out by dynamic light scattering (DLS) using a Zetasizer μV (Malvern Instruments Ltd). Measurements of a minimum of 13 data points at 293 K were taken in triplicate from enzyme solutions at 2.5, 5 and 10 mg ml⁻¹.

2.2. Crystallization

The purified lyophilized CEP was resuspended at a concentration of 10 mg ml⁻¹ in 50 mM Tris buffer pH 8.0. Crystallization screenings were carried out automatically with a Honeybee 931 crystalization robot (Genomic Solutions Inc.) using a variety of commercially available screens (Qiagen). Drops of 2 μl final volume (1:1 ratio of protein and mother liquor) were set up using the sitting-drop vapour-diffusion technique and maintained at a temperature of 291 K. Clusters of crystal plates grew in reservoir solutions containing ammonium sulfate, Tris buffer, polyethylene glycol monomethyl ether (PEG MME) 2000 and glycerol as a precipitant. Attempts at crystallization optimization were carried out in both hanging-drop and sitting-drop plates (24-well Linbro plates).

2.3. Data collection and processing

The cluster of plates was separated using an acupuncture needle and a single CEP crystal was transferred to a cryoprotectant with a composition similar to that of the reservoir solution but with the addition of 20% glycerol. The protein crystal was harvested with a cryoloop and flash-frozen directly in a nitrogen stream prior to X-ray data collection. The diffraction data were collected on beamline MX2 at the Synchrontron Light Source Laboratory (LNLS), Campinas, Brazil using a MAR CCD detector (Guinierat et al., 2009). A data set of 108° coverage was collected at a wavelength of 1.46 Å using the oscillation method with an angular range of 0.4°. Data integration and scaling were carried out using the program XDS (Kabsch, 2010). Molecular replacement was carried out with the program Phaser (McCoy et al., 2007) and model building and refinement were carried out with the programs Coot (Emsley et al., 2010) and REFMAC5 (Murshudov et al., 2011), respectively.

3. Results and discussion

The isolation and purification of the enzyme CEP from leaves of C. excelsa led to high yields of pure protein. Analysis of the purified CEP (theoretical molecular weight of 45 kDa) by native PAGE and SDS–PAGE showed a major single migration band at around 50 kDa (Fig. 1), demonstrating high purity and the absence of aggregates even at the higher protein concentration of 10 mg ml⁻¹. Consistent with the native gel analysis, DLS studies also demonstrated a monomodal and monodisperse distribution at all three CEP concentrations, with a calculated molecular weight of 99 ± 15 kDa and a polydispersity index of 14 on average. The experimentally observed higher molecular weight of the enzyme on SDS–PAGE is an indication that the protein is in a glycosylated form, which is in agreement with the predicted N-linked glycosylation sites based on the sequence motif N-X-S/T (where X is any amino acid except proline). According to our DLS experiments, it is likely that CEP forms dimers in solution.

Several crystallization hits were found from the >800 different conditions screened; however, only reservoir solutions containing ammonium sulfate were suitable for producing crystals with X-ray diffraction properties. Small crystal plates grew from condition No. 83 of the Cryos Suite crystallization kit (Qiagen). Further crystallization...
optimization led to the production of well sized reddish crystals using 0.17 M ammonium sulfate, 0.085 M Tris pH 8.0, 17% PEG MME 2000, 15% glycerol. Small crystal plates started appearing within one week of crystallization setup and reached their maximum size, with dimensions in the range 0.3–0.6 mm and 0.05–0.1 mm, in approximately 24 h. Despite their thin-plate morphology (Fig. 2), these crystals were suitable for X-ray data collection. A complete native data set was collected to a resolution of 2.6 Å on the dedicated wiggler beamline MX2 (LNLS, Brazil; Fig. 3). The crystal was assigned to the orthorhombic system, with space group \(P2_12_12_1\) and unit-cell parameters \(a = 70.2, b = 100.7, c = 132.3\) Å. Data-collection and processing statistics are shown in Table 1. According to the Matthews coefficient of 2.11 Å\(^3\) Da\(^{-1}\) (Matthews, 1968), the crystal solvent content is 42% considering two molecules in the asymmetric unit. Calculation of the self-rotation function revealed no additional peaks, as expected for twofold noncrystallographic symmetry (NCS) of both molecules in the asymmetric unit, suggesting an NCS axis parallel to one of the crystal axes (data not shown). Further native Patterson analysis showed a peak \((u, w) = (0, 0.38)\) in the Harker section \((u, 1/2, w)\), indicating an NCS axis parallel to the crystal axis \(c\) with a fractional translation vector of 0.38 (Fig. 4).

Structure determination of CEP was carried out by molecular replacement using the structure of peroxidase from the royal palm tree \(R. regia\) (PDB entry 1hdl; Watanabe et al., 2010) as a search model. The search model was manipulated for this purpose so that the covalently attached carbohydrates, the haem group and non-bonded atoms were not considered during the rotation and translation searches. A unique solution with an LL gain of 1193 and a \(Z\) score of 46 was obtained when searching for two molecules in the asymmetric unit. In addition to electron-density inspection, initial cycles of rigid-body and restrained refinement brought the primary solution to an \(R\) factor of 36.5%, an \(R_{	ext{free}}\) of 34.8% and a figure of merit (FOM) of 0.60 to values of 28.6%, 32.8% and 0.66, respectively, which are good indicators of a correct structure solution.

A preliminary inspection of the map shows clear additional electron density for the haem group (which was not included in the search model) and possible Ca atoms, which were presumably copurified together with the native protein, and at least nine glycosylation sites. The chemical nature of the sugar moieties is under analysis. Further crystallographic refinement and model building is currently in progress.

Table 1
Data-collection and processing statistics.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Beamline</td>
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<tr>
<td>Wavelength (Å)</td>
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<tr>
<td>Space group</td>
<td>(P2_12_12_1)</td>
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<tr>
<td>Unit-cell parameters (Å)</td>
<td>(a = 70.2, b = 100.7, c = 132.3)</td>
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<tr>
<td>Resolution range (Å)</td>
<td>80.1–2.6 (2.7–2.6)</td>
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<tr>
<td>No. of unique reflections</td>
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<tr>
<td>Mosaicity (%)</td>
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<tr>
<td>Multiplicity</td>
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<tr>
<td>Completeness (%)</td>
<td>90.1 (91.8)</td>
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<tr>
<td>(R_{	ext{merge}}) (%)</td>
<td>10.1 (55.3)</td>
</tr>
<tr>
<td>(\langle I/\sigma(I) \rangle)</td>
<td>10.4 (3.7)</td>
</tr>
</tbody>
</table>

\(\langle I/\sigma(I) \rangle = \frac{\sum_{\text{obs}} |I(hkl)| - \langle I(hkl) \rangle}{\sum_{\text{obs}} |I(hkl)|} / \sum_{\text{obs}} |I(hkl)|\), where \(I(hkl)\) is the observed intensity of an individual reflection and \(\langle I(hkl) \rangle\) is the average intensity of that reflection.

Figure 2
Crystals of CEP grown in 0.17 M ammonium sulfate, 0.085 M Tris pH 8.0, 17% PEG MME 2000 and 15% glycerol using the hanging-drop vapour-diffusion technique. The black bar corresponds to 100 μm.

Figure 3
(a) Diffraction pattern of a plate-like crystal of CEP extending to a resolution of 2.6 Å. (b) Enlarged view of the diffraction pattern with contrast enhancement.
4. Conclusions

The peroxidase from the leaves of the palm tree _C. excelsa_ was successfully extracted and purified in its native form. Experimental data strongly indicate that CEP is glycosylated, consistent with other class III peroxidases (Welinder _et al._, 1992). Diffraction-quality crystals were obtained from conditions containing ammonium sulfate and a native X-ray diffraction data set was collected at 2.6 Å resolution. A molecular-replacement solution was found and structure analysis awaits final model building and refinement.

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References


Figure 4

Harker section (ν = 0.5) of the native Patterson map at a resolution of 4 Å showing a large peak at (ν, τ) = (0, 0.38) corresponding to the intermolecular translation vector along e relating the two copies of the molecule in the asymmetric unit. The map was calculated with a minimum contour level of 1.5σ in intervals of 1.0σ using the CCP4 program suite (Winn _et al._, 2011).