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# Monoclinic crystals of lignin peroxidase

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The lignin peroxidase isozyme with an isoelectric point of 4.15 from *Phanerochaete chrysosporium* was crystallized and the crystal parameters were determined. The new crystals are monoclinic but they are related to the orthorhombic crystals previously obtained for another isozyme. Lignin peroxidase was produced in a semi-continuous process to obtain adequate amounts of protein for crystallization. Combining multiple harvests from such a production process did not interfere with crystallization.

Lignin peroxidase; Protein crystallization; Semi-continuous cultivation; (*Phanerochaete chrysosporium*)

## 1. INTRODUCTION

Lignin is a random 3-dimensional polymer based on phenylpropane units. It protects cellulose and hemicellulose from enzymatic degradation in plants and subsequently provides rigidity to vascular plants like trees [1]. Being the second most abundant renewable material on earth and rich in aromatic substructures lignin is likely to be an important substance for energy production and chemical supply [2,3]. Lignin can be degraded enzymatically through a combined action of several enzymes. The exact role of each enzyme is as yet not clear [4].

Lignin peroxidase (ligninase, LiP, EC 1.11.1.7) is a possible key enzyme involved in lignin breakdown (see [5] for a review) and the only lignin-modifying enzyme crystallized so far. Ligninase is produced by several wood-rotting fungi of which *Phanerochaete chrysosporium* is so far studied most.

LiP was first detected in culture fluids of *P. chrysosporium* in 1983 and was subsequently purified [6,7]. Since then, extensive research efforts to characterize this enzyme have been undertaken, e.g. [8–10]. Ligninase has been shown to be a glycoprotein of about 40 kDa and it carries one iron protoporphyrin IX as a prosthetic group. Probably the best known reaction catalyzed by ligninase is the conversion of veratryl alcohol to veratraldehyde, which is also used as an activity assay for ligninase [11]. LiP occurs in multiple isozymic forms [8,9].

Ligninases have been characterized by kinetic and spectroscopic methods [20] but detailed structural data are still lacking. However, one isozyme of ligninase (LiP 4.65) has already been crystallized [12] and the crystal structure determination of ligninase is in progress [13].

Here we describe the crystallization of a second isozyme of ligninase (LiP 4.15) which differs in catalytic properties and stability from LiP 4.65. It is produced in much smaller amounts by *P. chrysosporium* as LiP 4.65. As an overproduction system for lignin peroxidase in *P. chrysosporium* is not yet available, we have used semi-continuous cultivation and combined the harvests to obtain sufficient protein for crystallization experiments. Batch combination is not the usual approach to this quantity problem in protein crystallization but our results show that it can be used if sufficiently pure material can be obtained.

## 2. EXPERIMENTAL

### 2.1. *Production of lignin peroxidase*

Lignin peroxidase was produced by cultivating *P. chrysosporium* BKM-F-1767 (ATCC 24725) in C-limited cultures in shake flasks as described in [14]. Cultures were induced with veratryl alcohol for ligninase production 72 h after inoculum. Harvesting was done usually about 48 h after induction by collecting the culture fluids by filtration. When semi-continuous cultivation was used, fungal pellets were not discarded but redissolved in about 150 ml of glucose-free medium and LiP production induced again. Subsequent harvesting followed 48 or 72 h after re-induction. Up to four harvesting cycles could be made. Each harvest was ultrafiltrated (10 kDa cut-off membrane) individually to a volume of about 1 ml right after harvesting and stored at 4°C until the cultivation was stopped.

### 2.2. *Purification and crystallization of lignin peroxidase*

Sequential harvests were pooled prior to further purification.

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*Abbreviations:* LiP 4.15, ligninase isozyme with the isoelectric point of 4.15; LiP 4.65, ligninase isozyme with the isoelectric point of 4.65; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; IEP, isoelectric point

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Ligninase was then purified by preparative-scale flat-bed isoelectric focussing and gel filtration as described earlier [12] with 20 mM acetic acid, pH 3.3, as eluent in gel filtration. Crystals were grown under similar conditions as before [12] in hanging drops by the vapour diffusion technique using 10 μl drops over a 1 ml precipitant solution in plastic trays (Limbro) [15]. Seedings were carried out by first crushing crystals (either badly shaped or too small for further work) in the precipitant solution with a glass hair to submicroscopic pieces (not visible with 100× magnification). Various volumes (usually much less than 1 μl) were then introduced into drops, which were supersaturated with respect to crystalline LiP, with a thin glass pipette to promote crystallization.

2.3. *Analytical methods*

Purity and reproducibility of the protein preparations were checked by SDS-PAGE and analytical isoelectric focussing against standard proteins [16], carbohydrate content estimation using the orcinol test (mannose standard) [17] and by specific activity determination [11].

2.4. *Crystallographic methods*

Crystals were mounted in glass capillaries and sealed between small amounts of mother liquor [15]. Precession photographs were recorded on an Enraf-Nonius precession camera with nickel-filtered Cu-$K_\alpha$ X-radiation from a sealed-off tube (AEG, super fine focus, 40 kV, 20 mA). The exposure time for one 11° screened precession photograph was 40 h. Crystal to film distance was 75 mm.

## 3. RESULTS

### 3.1. *Production and purity of crystallizable LiP*

Table 1 summarizes the content of LiP in sequential harvests. In this case only the first three cycles were pooled for enzyme purification, because the crude ligninase from cycle 4 had a much lower specific activity. Before the different harvests were combined, samples were taken to detect possible changes in isozyme pattern during cultivation. The protein pattern obtained by analytical isoelectric focussing (fig.1) shows that LiP 4.15 and LiP 4.65 are produced throughout. In contrast, two proteins with an isoelectric point around 4.3 can be seen for which the relative ratio changes with time.

In all cases LiP 4.15 after purification shows only one band both in isoelectric focussing (fig.1) and in SDS-PAGE (fig.2). The molecular mass as estimated from SDS-PAGE is 41 kDa. The specific activity of the pure LiP 4.15 was 22–24 U/mg protein. LiP 4.15 was found to contain 5–10 times more sugars (w/w) associated with the protein preparation than LiP 4.65 as judged by orcinol tests for several samples from different cultivations. LiP 4.65 contains constantly around 15% carbohydrate (w/w).

Table 1

Semi-continuous production of lignin peroxidase from *P. chrysosporium*

| Harvest cycle | Volume (ml) | Total protein (mg) | Total ligninase (U) | Specific activity (U/mg) | U/l |
|---|---|---|---|---|---|
| 1 | 840 | 9.0 | 77.1 | 8.6 | 91.8 |
| 2 | 1370 | 17.1 | 129.1 | 7.6 | 94.2 |
| 3 | 1420 | 13.9 | 108.7 | 7.8 | 76.5 |
| 4 | 1030 | 9.2 | 52.8 | 5.8 | 51.2 |

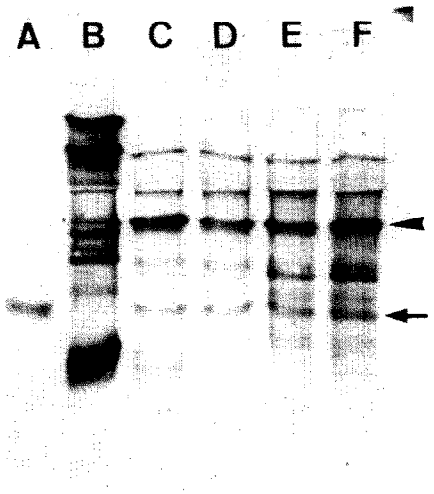


Fig.1. LiP isozyme pattern on an analytical isoelectric focussing gel (pH range 3–5.5) of four successive harvests of a semi-continuous enzyme production. Samples are (A) LiP 4.15 after purification; (B) standard proteins; (C–F) 1st–4th harvest, respectively. The arrowhead points to pH 4.65 and the arrow to pH 4.15.

### 3.2. *Crystallization and crystal characterization*

Crystals were obtained in the hanging drops after several months of vapour diffusion equilibration (fig.3A). The final polyethyleneglycol concentration in the precipitant reservoirs was then 35% (w/v). More crystals could easily be obtained by seeding with submicroscopic sized crystals in only 1–2 days (fig.3B). Crystals are plate-like rhombohedrons, the third dimension seldom exceeding one fifth of the other two (fig.3C). The characteristic brown colour of ligninase (due to the haem group) is retained in the crystals.

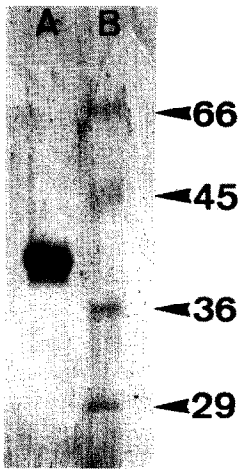


Fig.2. SDS-PAGE (10% gel) of purified LiP 4.15 (A) and standards (B). Numbers represent kDa. Estimated molecular mass for LiP 4.15 is 41 kDa.

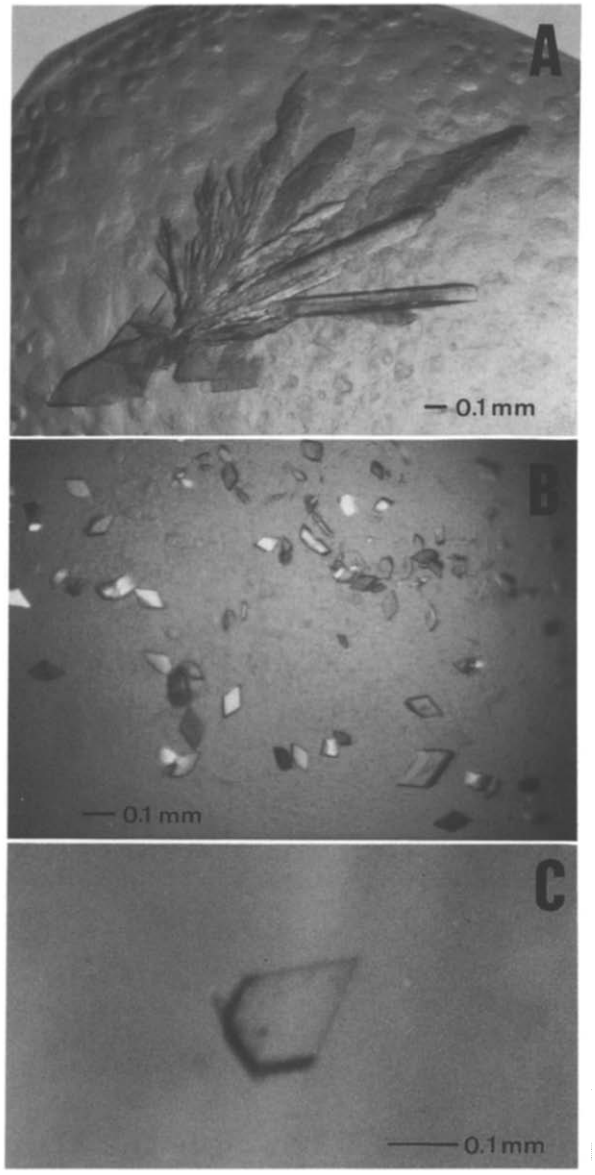


Fig.3. Crystals of LiP 4.15. (A) De novo crystals; (B,C) crystals from seeding experiments.

Strong birefringence in polarized light (fig.3B) and sharp edges further identify the material as crystalline. Maximum crystal dimensions of about 0.5 × 0.5 × 0.15 mm could be achieved.

In the seeding experiments it was very difficult to induce only a few crystals per drop but mostly at least one crystal reached a size sufficient for examination by X-ray diffraction. From precession photographs of the principal zones *h0l*, *0kl* and *hk0*, LiP 4.15 proved to have crystallized in the monoclonic space group $P2_1$ and the unit cell dimensions could be determined (table 2).

Intergrown crystals and twinning in the monoclinic *h0l* zone posed a more serious problem, as this caused many well-sized crystals to be useless for camera work.

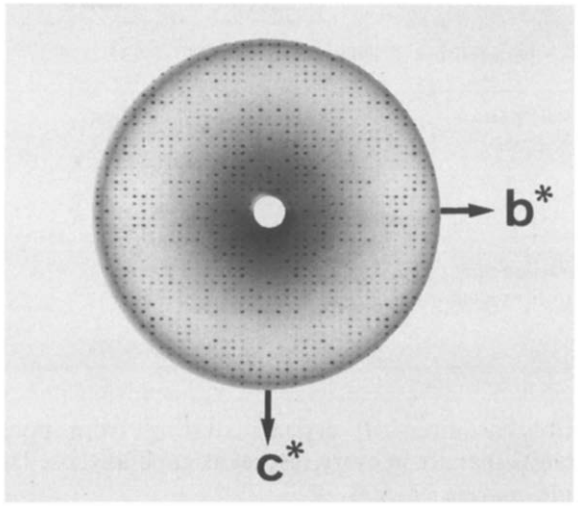


Fig.4. Precession photograph (*0kl*-zone, $\mu$ = 11°) of LiP 4.15.

The ligninase crystals seem to be very stable in the X-ray beam (up to about 200 h).

## 4. DISCUSSION

Monoclinic crystals of lignin peroxidase isozyme with IEP = 4.15 from *P. chrysosporium* were grown by vapour diffusion equilibration and characterized (table 2). The unit cell is clearly related to that of the orthorhombic crystal modification of a previously crystallized isozyme (LiP 4.65: $P2_12_12_1$; $a$ = 41.6, $b$ = 73.0, $c$ = 109.5 (Å), one molecule per asymmetric unit) [12] although their crystal morphology is strikingly different, i.e. plate-like rhombohedrons versus needles. The packing of the molecules in the two crystal modifications must be closely related as well: the diffraction pattern of the monoclinic crystals reveals a pseudo-orthorhombic $2_12_12_{(1)}$-packing of ligninase molecules in the monoclinic lattice [13]. However, the enzymatic properties of these two crystallized ligninases are very different in terms of catalytic activity, stability and substrate specificity [20]. It is conceivable that the two crystallized isozymes could play different roles in the enzymatic breakdown of lignin. Thus, the crystallization of LiP 4.15 next to LiP 4.65 opens the possibility to determine a second crystal structure of ligninase and ultimately, to gain better insight in the biological breakdown of lignin.

In protein crystallography it is very important that sufficient crystallizable material is readily available. This requirement is not fulfilled with LiP 4.15. The only way to meet this condition was to pool successive harvests from a semi-continuous shake flask culture to obtain adequate amounts of protein at a reasonable time/profit ratio. This approach can lead to unsatisfactory results in crystallization in the form of increased microheterogeneity [18]. However, we can repro-

Table 2

Characteristics of lignin peroxidase IEP = 4.15 crystals

| | |
|---|---|
| Crystal system | monoclinic |
| Space group | $P2_1$ |
| *a* | 44.9 (± 0.1) Å |
| *b* | 77.3 (± 0.2) Å |
| *c* | 99.4 (± 0.2) Å |
| $\beta$ | 100.9° |
| Diffraction limit | 2.6 $Å^{-1}$ |
| Unit cell volume | 338800 $Å^3$ |
| Z | 4 |
| $V_M$ | 2.07 $Å^3$/D |

ducibly acquire LiP crystals starting from pooled harvests that are in every respect as good as those from single enzyme batches.

In semi-continuous shake flask cultures usually only the first three harvests were pooled, because the cultures did not remain free of contaminations after that and the total ligninase yield diminished considerably (table 1). Both costs (less culture medium needed) and the time required to produce ligninases from *P. chrysosporium* could be reduced considerably by pooling multiple harvests as compared to three single cultivations (always starting from the primary growth phase of the fungus). We have also tried to crystallize LiP 4.15 from preparations produced in larger amounts in a bioreactor. These experiments were never successful. The reason might reside in the slightly different culture conditions as the buffer used in shake flask cultivation, 2,2-dimethylsuccinic acid, is too expensive and therefore omitted from large scale enzyme production.

Isoelectric focussing is a rapid and powerful method for preparing pure protein for crystallographic purposes. Attempts to crystallize crude ligninase (a mixture of many isozymes [8]) have, as expected [19], not been successful. This is well understood, since we have shown that ligninase isozymes from *P. chrysosporium* differ in molecular mass, carbohydrate content and amino acid composition [13,20]. It clearly makes them different items in terms of incorporation in a crystal lattice.

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