Isolation and Characterization of Wild-Type Lipoxygenase $LOX_{Psa}1$ from *Pleurotus sapidus*

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The lipoxygenase $LOX_{Psa}1$ of *Pleurotus sapidus*, originally investigated because of its ability to oxidize (+)-valencene to the valuable grapefruit aroma (+)-nootkatone, was isolated from the peptidase-rich lyophilisate using a three-step purification scheme including preparative isoelectric focusing and chromatographic techniques. Nano-liquid chromatography electrospray ionization tandem mass spectrometry (nLC-ESI-MS/MS) of the purified enzyme and peptide mass fingerprint analysis gave 38 peptides of the lipoxygenase from *P. sapidus*. Nearly 50 % of the 643 amino acids long sequence encoded by the cDNA was covered. Both terminal peptides of the native $LOX_{Psa}1$ were identified by *de novo* sequencing, and the postulated molecular mass of 72.5 kDa was confirmed. With linoleic acid as the substrate, the $LOX_{Psa}1$ showed a specific activity of 113 U mg^{-1} and maximal activity at pH 7.0 and 30 °C, respectively.

Key words: Basidiomycete, Lipoxygenase, *Pleurotus sapidus*

Introduction

The edible fungus *Pleurotus sapidus* is a member of the phylum Basidiomycota, which encloses about 30,000 species (Kirk *et al.*, 2008). Around 1000 thereof are appreciated as a delicious and nutritious food. Owing to the increasing demand for renewable natural sources of bioactive compounds and enzymes suitable for biotechnological and food applications, many recent studies have focused on basidiomycota (Alarcon *et al.*, 2006; Gruhn *et al.*, 2007). According to their capability to degrade lignified biopolymers effectively, basidiomycota produce a unique set of enzymes comprising oxidoreductases, such as laccases, lignin peroxidases, manganese peroxidases, as well as peptidases, glycoside hydrolases, esterases, and other hydrolases (Bouws *et al.*, 2008; Zorn *et al.*, 2005). However, work on enzymes from basidiomycota is often complicated by interfering activities, such as laccases which catalyze the polymerization of phenols and were found to interfere with the standard assay of fungal ferulic acid esterases (Haase-Aschoff *et al.*, 2013). As these organisms are a rich source of peptidases (Linke *et al.*, 2008), the rapid peptidolytic degradation of proteins of interest is another fundamental obstacle.

P. sapidus catalyzes the allylic oxidation of terpenoic compounds, such as (+)-valencene to (+)-nootkatone (Fraatz *et al.*, 2009), *R*-(+)-limonene to *R*-(–)-carvone (Kaspera *et al.*, 2005), α-pinene to verbenone (Krings *et al.*, 2009), and (+)-car-3-ene to car-3-ones/ols (Lehnert *et al.*, 2012) in high yields. Guided by the valencene oxidizing activity, an enzyme called ValOx was purified and characterized as a lipoxygenase (LOX) by reaction mechanism and sequence homology. According to the peptide sequences obtained by mass spectrometry, a cDNA sequence encoding a polypeptide of 396 amino acids was initially postulated (Fraatz *et al.*, 2009).

However, a homology comparison with the meanwhile available genome of the closely related *Pleurotus ostreatus* (oyster mushroom) appeared to indicate that 247 additional N-terminal amino acids were missing. Recently, the full sequence was functionally expressed in *Escherichia coli* (Zelena *et al.*, 2012) and the protein biochemically characterized (Plagemann *et al.*, 2013). Although the recombinant enzyme $LOX_{Psa}1$ showed

good LOX activity, the yields of conversion of valencene were significantly lower compared to those obtained with the native enzyme [80 *vs*. 280 mg (+)-nootkatone L^{-1}] (Krügener *et al.*, 2009; Zelena *et al.*, 2012). Thus, it was decided to isolate enough $LOX_{Psa}1$ from the lyophilisate of *P. sapidus* to fully characterize the native enzyme. After numerous failures, a purification scheme coping with the abundant peptidolytic activities of lyophilisates of basidiomycetes was eventually developed.

Materials and Methods

Fungus

Pleurotus sapidus (DSMZ 8266) was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. For maintenance on agar slants and submerged culture, the fungus was grown on glucose/L-asparagine/yeast extract medium as described elsewhere (Onken and Berger, 1999). Production of biomass and subsequent lyophilization were performed as described by Krings *et al*. (2009). Briefly, *P. sapidus* was cultivated in shaking flasks, and biomass (25 g dry matter L^{-1}) was harvested 5 d after inoculation.

Purification of active enzyme

Ten g of lyophilized biomass were re-suspended in 100 mL Tris-HCl (20 mM, pH 7.5) supplemented with Protease Inhibitor Mix FY (Serva, Heidelberg, Germany) and extracted for 1 h at 4 °C by agitation in horizontal position in an orbital shaker. Subsequently, insoluble components were removed by means of centrifugation (20 min, 6000 × *g*, 4 °C), and the supernatant was subjected to preparative isoelectric focusing.

Preparative isoelectric focusing (pIEF)

The supernatant was mixed with 2% Servalyt (pH 3 – 6; Serva) and transferred to the focusing chamber of a Rotofor cell (Bio-Rad, Munich, Germany). The chamber was cooled to 2 °C. Focusing was carried out at constant power of 12 W using 0.5 M ethanolamine and 0.5 M acetic acid as anode and cathode buffers, respectively. After 5 h, 20 fractions were harvested. Of each fraction, the pH value was measured, and the LOX activity was determined spectrophotometrically by monitoring the increase in absorbance at 234 nm during the transformation of linoleic acid (Plagemann *et al.*, 2013). Fractions with LOX activity were pooled, diluted with distilled water to a final volume of 200 mL, and subjected to anion exchange chromatography.

Ion exchange chromatography (IEX)

Anion exchange chromatography was performed on a HiTrap Q-XL column (1 mL; GE Healthcare, Freiburg, Germany) using potassium phosphate (10 mM, pH 6.5) as a start buffer (buffer A). Proteins were eluted with a step gradient of potassium phosphate containing 1 M sodium chloride (buffer B) over four steps: 10 column volumes (CV) 100% buffer A, 10 CV 90% buffer A, 10 CV 80% buffer A, 10 CV 0% buffer A. The flow rate was 1.0 mL min^{-1} and the fraction size was 2 mL. Fractions with LOX activity were pooled, concentrated to 250 μL by ultrafiltration, and subjected to size exclusion chromatography.

Size exclusion chromatography (SEC)

For SEC, a Superdex 75 10/300 GL column (24 mL; GE Healthcare) with an optimum separation range of 3 – 70 kDa was employed. Tris-HCl (20 mM, pH 7.5) served as elution buffer. The flow rate was adjusted to 0.3 mL min^{-1}, and the fraction size was 1 mL.

Gel electrophoresis

The purity of protein samples was monitored using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% (w/v) polyacrylamide gels (Laemmli, 1970). Proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Serva). To determine molecular masses, unstained standard proteins (Precision Plus Protein; Bio-Rad) were used.

Enzyme activity

To determine the LOX activity, the increase in absorbance at 234 nm caused by LOX-induced transformation of linoleic acid to the corresponding conjugated diene hydroperoxides was measured spectrophotometrically. The assay was performed as described elsewhere (Plagemann *et al.*, 2013).

Determination of pH and temperature optima

The temperature optimum was determined in the range of 20 – 50 °C using sodium phosphate buffer (50 mM, pH 7). The enzyme-buffer mixture was tempered at the appropriate temperature for 5 min prior to the addition of the substrate. Determination of the pH optimum was carried out at 30 °C using Britton-Robinson buffer (Britton and Robinson, 1931) in the range of pH 4 – 9.5.

LOX identification

Assigned LOX bands were excised from SDS polyacrylamide gels, dried, consecutively incubated with dithiothreitol and iodine acetamide, and finally digested with trypsin (sequencing grade modified trypsin; Promega, Madison, WI, USA). The resulting peptides were extracted and purified according to standard protocols. Peptides, re-dissolved in 20 μL H_2O, 2% (v/v) acetonitrile, and 0.1% (v/v) formic acid were analysed using the nano-liquid chromatography (nLC) system EASY-nLC II (Bruker Daltronik, Bremen, Germany) equipped with a 20-mm pre-column (C18-A1 3PCS; ThermoFisher Scientific, Dreieich, Germany) and a capillary column (0.1 mm × 150 mm) packed with Magic C18 AQ (3 μm particle size, 200 Å pore size; Michrom Bioresources, Inc., Auburn, CA, USA) eluted by a linear gradient (300 nL min^{-1}) of water and acetonitrile [each with 0.1% formic acid (v/v)] from 95% water to 95% acetonitrile within 25 min and hold for 15 min. The nLC system was connected to a maXis impact QTOF mass spectrometer (Bruker Daltronik) equipped with a captive nanospray ion source for electrospray ionization in the positive mode. For collision-induced dissociation experiments, multiple-charged parent ions were automatically transmitted from the quadrupole mass analyzer into the collision cell (21 – 50 eV). The resulting daughter ions were separated by an orthogonal time-of-flight (TOF) mass analyzer operating with lock mass calibration (1221.9906) and mass resolution > 30,000. Peptide mass fingerprint analysis was done with ProteinScape 3.0 (Bruker Daltronik) and applied for cross-species protein identification (NCBInr, current in-house releases) using the Mascot software server (Mascot 2.4.0 search engine; Matrix Science, London, UK). Parameters were set as follows: enzyme, trypsin/P; global modification, carbamidomethylation (variable); oxidation (M, variable), up to 1 missing cleavage allowed; precursor ion mass tolerance, 10 ppm; fragment ion mass tolerance, 0.05 Da; peptide charge, 1+, 2+, 3+; instrument, ESI QUAD TOF (Bruker Daltronik); minimum peptide length, 4; Mascot score, > 30.

Results and Discussion

In order to isolate the native LOX completely and in active form from lyophilized mycelia of *P. sapidus*, in spite of the presence of a multitude of concomitant enzymes partly known to exhibit peptidolytic activities, a three-step purification scheme was developed (Table I). After the rehydration of the lyophilisate, the water-soluble components were separated according to their isoelectric points (pI) using pIEF. In agreement

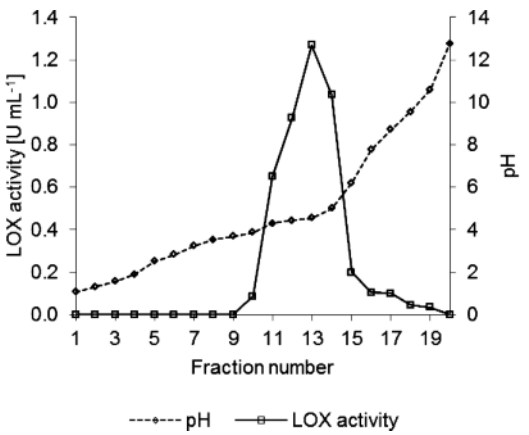

Fig. 1. Preparative isoelectric focusing of a crude extract from *Pleurotus sapidus*.

Table I. Summary of the purification of $LOX_{Psa}1$ from *Pleurotus sapidus*.

Purification step	Protein [mg]	Specific activity [U mg^{-1}]	Recovery (%)	Purification (*x*-fold)
Crude extract	436.9	0.403	100.0	1.0
pIEF	169.9	0.471	45.5	1.2
IEX	6.9	9.91	38.8	24.6
SEC	0.1	113.05	6.4	280.3

Fig. 2. SDS-PAGE of the purified LOX from *Pleurotus sapidus* after three-step purification. Lane M, Precision Plus Protein™ Standard All Blue; lane S, SEC fraction, showing LOX activity.

with the pI reported for a purified LOX from *Pleurotus ostreatus* (Kuribayashi *et al.*, 2002), the present study determined a pI of 4.5 – 5 by measuring the pH value of each fraction (Fig. 1).

Fractions 10 to 15 were pooled and subjected to IEX on a strong anion exchanger. The main LOX activity was found in fractions 13 to 18 eluted with 0.2 M sodium chloride.

Subsequently, the active fractions were pooled again and separated by SEC, which yielded one LOX-active fraction. Analysing the purified enzyme by means of SDS-PAGE, a single band corresponding to a molecular mass of approximately 75 kDa was obtained (Fig. 2).

The Coomassie-Brilliant Blue R-250-stained band was carefully excised from the gel and submitted to tryptic digestion with subsequent identification of peptides by nano-liquid chromatography quadrupole time-of-flight tandem mass spectrometry (nLC-QTOF-MS/MS). A total of 38 peptides of the LOX of *P. sapidus* (gi/466860334) were identified which resulted in a Mascot score of 2700. The sequence coverage was 48.5 % (Fig. 3). Unambiguous identification of two peptides "VHNISLSSR" (missing only the N-terminal amino acid) and "LAAVFLVNSAQLDDQNTPYDVLAPEQLANAIVI" (including the typical isoleucine C-terminus) confirmed a molecular mass of 72,506 Da of the native LOX (amino acids). Hence, the formerly annotated valencene oxygenase from *P. sapidus* ValOx (Fraatz *et al.*, 2009) is not a related dioxygenase or an isoenzyme, but rather a smaller fragment of the LOX formed by peptidolysis during the LOX purification procedure. Although a peptidase inhibitor mix was added in all steps of the present work, the SDS-PAGE gel still showed two additional weak bands with smaller molecular masses. The tryptic peptide sequences found for these gel bands fitted without any exception to the sequence of the LOX.

MVHNISLSSRKALHNVHLPYMVQLPKPTGYNVALKNAAEGYDKARRMVAWL

YDIADYESSIPQTFTLQQKTDKYTWELSDNFPPHLAVVPPDQSVSAPSIFSPVRL

AQTLLIMSSLWYDDHTDLAPGPEQNTMQKLTQWNQERHKDQGWLIKDMFNA

PNIGLRNDWYTDEVFAQQFFTGPNSTTITLASDVWLTAFTSEAKAQGKDKVIAL

FESAPPNSFYVQDFSDFRRRMGAKPDEELFNDSDGAMRYGCAAVALFYLTAM

GKLHPLAIIPDYKGSMAASVTIFNKRTNPLDISVNQANDWPWRYAKTCVLSSD

WALHEMIIHLNNTHLVEEAVIVAAQRKLSPSHIVFRLLEPHWVVTLSLNALARS

VLIPEVIVPIAGFSAPHIFQFIRESFTNFDWKSLYVPADLESRGFPVDQLNSPKFH

NYAYARDINDMWTTLKKFVSSVLQDAQYYPDDASVAGDTQIQAWCDEMRSG

MGAGMTNFPESITTVDDLVNMVTMCIHIAAPQHTAVNYLQQYYQTFVPNKPSA

LFSPLPTSIAQLQKYTESDLMAALPLNAKRQWLLMAQIPYLLSMQVQEDENIVT

YAANASTDKDPIIASAGRQLAADLKKLAAVFLVNSAQLDDQNTPYDVLAPEQL

ANAIVI

Fig. 3. Sequence coverage of native LOX. Amino acids present in the sequenced 38 tryptic peptides are underlined.

The LOX-catalyzed transformation of linoleic acid to the respective conjugated diene hydroperoxide as a function of pH value and temperature was determined spectrophotometrically using the purified enzyme. In accordance with the recombinant $LOX_{Psa}1$, the specific activity of the native enzyme was 113 U mg^{-1}. The optima were found at 30 °C and pH 7 (Fig. 4), respectively. Both results agree with the optima determined for the recombinant $LOX_{Psa}1$, which were 25 – 35 °C and pH 7, respectively (Plagemann *et al.*, 2013). Similar ranges of maximal activity have been reported for other fungal LOXs. pH optima of LOXs produced by the basidiomycete *Pleurotus ostreatus* or the ascomycetes *Thermomyces lanuginosus* and *Gaeumannomyces grammini*, which excrete a manganese LOX, were in the range of pH 6 – 8 (Knapp *et al.*, 2001; Kuribayashi *et al.*, 2002; Su and Oliw, 1998). The temperature optima of LOXs from *Pleurotus ostreatus* and the ascomycete *Botryodiplodia theobromae* were found at 25 °C and 30 °C, respectively (Kuribayashi *et al.*, 2002; Gonzalez *et al.*, 2003).

There are only very few studies on purified LOXs from basidiomycota. Kuribayashi *et al.* (2002) isolated and characterized a LOX from *Pleurotus ostreatus*. Although the amino acid sequence of the enzyme showed high similarities to $LOX_{Psa}1$, the optima of the purified native enzyme were pH 8 and 25 °C, respectively, and the enzymatic activity was significantly lower (23.4 U mg^{-1}) (Tasaki *et al.*, 2013).

Application of a purification scheme involving rigorous peptidase inhibition enabled the identification of the entire amino acid sequence. Although the wild-type enzyme had previously been assumed to comprise 369 amino acids only (Fraatz *et al.*, 2009), a sequence coding for 643 amino acids was postulated at the genetic level. The present work confirmed the accuracy of the recombinant $LOX_{Psa}1$ by identifying 38 peptides resulting in a sequence coverage of nearly 50 %. Accordingly, the wild-type $LOX_{Psa}1$ purified from the lyophilisate of *P. sapidus* on the one hand, and the enzyme produced heterologously in *E. coli* on the other hand, shared identical biochemical parameters (Zelena *et al.*, 2012; Plagemann *et al.*, 2013). Obviously, the lower valencene oxidizing activity of the recombinant protein, generated from the respective cDNA of *P. sapidus*, cannot be explained by a difference in the amino acid sequence. As the ESI-QTOF results did not suggest any particular post-translational modification of the native enzyme, a stimulating effect of unknown constituents of the lyophilisate is currently the best explanation of the observed differences in activity.

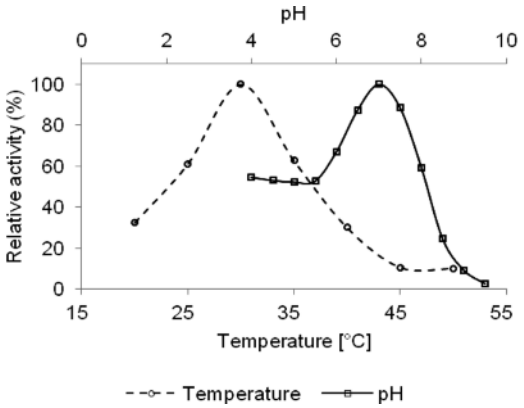

Fig. 4. Effect of temperature and pH value on the activity of the purified LOX.

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