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$LOX_{Psa}1$, the first recombinant lipoxygenase from a basidiomycete fungus

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ABSTRACT

A dioxygenase from the edible basidiomycete *Pleurotus sapidus*, originally researched because of its distinct ability to convert the sequiterpene (+)-valencene to the valuable grapefruit aroma (+)-nootkatone, was identified as a potent lipoxygenase ($LOX_{Psa}1$). Kinetic parameters, pH and temperature optima of the pure recombinant enzyme were determined using linoleic acid as the substrate. K_m, v_{max}, and k_{cat} were 40.3 μM, 130.3 U mg^{-1}, and 157 s^{-1}, respectively. The maximal enzymatic activity was found at pH 7.0 and 35 °C. Showing high specificity toward free linoleic acid, the enzyme was classified as lipoxygenase type 1. Conversion of linoleic acid yielded mainly (*S*)-13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (94% ee), as was confirmed by chiral HPLC analysis of the hydroperoxides. The amino acid sequence showed homology to lipoxygenases catalyzing *S* stereospecific oxygenation, and thus the enzyme was characterized as a 13*S*-lipoxygenase. This is the first lipoxygenase described to accept terpene hydrocarbons as substrates.

1. Introduction

Lipoxygenases (LOX) constitute a family of non-heme iron containing dioxygenases which catalyze the regio- and stereospecific insertion of molecular oxygen in polyunsaturated fatty acids (PUFA) with at least one (1*Z*,4*Z*)-pentadiene unit [1]. Forming highly reactive conjugated fatty acid hydroperoxides this reaction plays an important role as initial step in biosynthesis of a large variety of different products. LOX-derived metabolites, the so-called oxylipins, occur ubiquitous in plants [2], mammalians [3], fish [4], microorganisms [5] and fungi [6,7], performing a wide range of biological functions. In plants, they are involved in the regulation of development processes and defense against herbivore or microbial pathogen attack [8], whereas mammalian oxylipins, such as leukotrienes and lipoxins, regulate immune responses [9].

Owed to the formation of highly reactive compounds, LOXs are also of interest to the food industry. A number of flavor components including the green notes of fruits and vegetables can be formed along the enzymatic cleavage of fatty acid hydroperoxides into short chain aldehyds and alcohols [10]. Some types of LOX, such as LOX-1 from soybean, were found to catalyze co-oxidative reactions in the presence of PUFAs [11]. They are applied in breadmaking for the bleaching of carotenoids [12] and the improvement of dough rheology [13].

Although the occurrence of LOX activity in fungi and the associated biosynthetic pathway leading to the characteristic flavor compounds 1-octen-3-ol and 1-octen-3-one was suggested years ago, there are virtually no reports that C8 compounds originate from the activity of a fungal LOX [6,8]. Recent studies confirmed LOX activity in extracts of several fungal species, such as *Geotrichum candidum* [14], *Mortierella sp.* [15], *Penicillium camemberti* [16], *Penicillium roqueforti* [16] and *Morchella esculenta* [17]. In addition, LOXs of *Saprolegnia parasitica* [18], *Gaeumannomyces graminis* [7,19], *Pleurotus ostreatus* [6], *Tefezia claveryi* [20,21] and *Thermomyces lanuginosus* [22] were purified and partially characterized. Among them the ascomycete *G. graminis* ('take all' plant disease) excreted the most unusual fungal LOX – a so-called manganese LOX (Mn-LOX). Containing catalytic manganese instead of iron, this LOX showed remarkable differences in reaction mechanism and products compared to classical LOXs [23]. Mn-LOX is the only fungal LOX so far, which has been cloned and expressed in *Pichia pastoris* recombinantly [19,24].

From basidiomycota, a LOX from *P. ostreatus* has been isolated and biochemically characterized [6]. However, the sequence of this enzyme is still unknown, although the database of National Center for Biotechnology Information (NCBI) includes an mRNA-sequence of a LOX from *P. ostreatus* (GenBank: AB472334.1). Fraatz et al. [25] described the bioconversion of the sesquiterpene (+)-valencene to (+)-nootkatone by an oxygenase, provisorily termed valencene oxygenase (ValOx), from *Pleurotus sapidus*. According to the products obtained and homology data from partial sequences a lipoxygenase-like type of enzyme was postulated [26]. Recently, this valencene dioxygenase from *P. sapidus* was functionally expressed in *Escherichia coli* [27]. The aim

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of this study was to verify and characterize the recombinant enzyme.

2. Materials and methods

2.1. Chemicals

The purified recombinant enzyme was obtained as described by Zelena et al. [27]. Tris–HCl (>99%), citric acid monohydrate (>99%), formic acid (>98%), anhydrous sodium sulfate (>99%) and disodium phosphate (>99%) were purchased from Roth (Karlsruhe, Germany); linoleic acid (>99%, hydroperoxide-free) and sodium borohydride (>99%) were from Sigma–Aldrich (Taufkirchen, Steinheim, Germany); boric acid (>98.5%), iron(II) chloride tetrahydrate (>98%) and LOX-1 from soybean were provided from Fluka (Seelze, Germany). All other chemicals used were analytical grade.

2.2. Protein concentration

The protein concentration was determined by the method of Lowry [28] using *DC*-Protein-Assay (Bio-Rad, Munich, Germany) and bovine serum albumin as a standard. In the concentration range used (0.2 mg mL^{-1}–1.2 mg mL^{-1}) the calibration curve was linear with a coefficient of determination of $R^2 = 0.992$.

2.3. SDS-PAGE and Western blotting

The protein samples were diluted 1:2 with SDS loading buffer and denatured for 10 min at 95 °C. SDS-PAGE was performed according to [29] using 12% (w/v) polyacrylamide gels. Proteins were stained with 0.1% (w/v) Coomassie Brillant Blue R-250 (Serva, Heidelberg, Germany). Unstained standard proteins (Bio-Rad, Munich, Germany) were used for the preparation of a calibration curve for the determination of molecular masses.

For Western blot analysis the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The recombinant dioxygenase was detected using a Penta-His HRP conjugated Kit (5 PRIME, Hamburg, Germany). Staining was carried out using 4-chloro-1-naphtol and H_2O_2 according to the manual instructions.

2.4. Peptidolytic cleavage of the His-tag

To eliminate the His-tag epitope from the recombinant $LOX_{Psa}1$, 50 μg of the purified protein preparation were mixed with 1 μg Factor Xa Protease (NEB, Frankfurt am Main, Germany) in Factor Xa-Buffer (Tris/HCl 20 mM, NaCl 100 mM, $CaCl_2$ 2 mM, pH 8.0) and incubated for 6 h at 23 °C. A specific inactivation of the peptidase after the cleavage was omitted.

2.5. Enzyme activity

Enzyme activity was determined spectrophotometrically by monitoring the increase in the absorbance at 234 nm due to the transformation of linoleic acid to the respective conjugated hydroperoxydienes [30]. The assay was carried out in an UV-transparent 96-well microtiter plate containing 5 μL enzyme solution (0.1 ng purified enzyme μL^{-1} per well) and 175 μL citrate-phosphate buffer (pH 7; except determination of pH optima). The reaction was initiated by adding 40 μL of a freshly prepared 2 mM substrate solution, which was made of 20 μL (18 mg) linoleic acid, 30 μL (33 mg) Tween 20, and 60 μL 1 M NaOH in a 2 mL volumetric flask and filled up with distilled water. This mixture was diluted with distilled water to the appropriate final concentration of 2 mM linoleic acid (except kinetic studies). Absorbance at 234 nm was recorded every 30 s and monitored at least for 20 min using a microplate reader (Synergy 2, Biotek, Bad Friedrichshall, Germany), which was tempered to 22 °C (except determination of temperature optimum).

Enzyme activity was calculated on the basis of the molar extinction coefficient of the conjugated diene hydroperoxides at 234 nm ($\varepsilon = 2.5 \times 10^4\ M^{-1}\ cm^{-1}$). Blanks were carried out using 40 μL buffer instead of enzyme solution. All experiments were performed in triplicate.

2.5.1. Effect of pH and temperature on enzyme activity

The pH optimum was determined at 22 °C using McIlvaine citrate–phosphate buffer (0.1 M citric acid and 0.2 M Na_2HPO_4) for pH 4.0–8.0 and 100 mM borate buffer for pH 8.0–9.5. Determination of the optimal temperature was performed in the range of 22–50 °C using 182.4 mM citrate-phosphate buffer (pH 7). The addition of the substrate solution was not carried out until the enzyme-buffer mixture was tempered at the appropriate temperature for 5 min.

2.5.2. Study of kinetic parameters

Estimation of the Michaelis–Menten constant (K_m) and the maximum activity rate (v_{max}) was based on the method of Lineweaver and Burk [31] using various substrate concentrations (0.1–1 mM). Each experiment was carried out with enzyme solutions of three different concentrations (0.075 ng μL^{-1}, 0.1 ng μL^{-1}, 0.15 ng μL^{-1}).

2.6. Chemical characterization of reaction products

To characterize reaction products arising from bioconversion of linoleic acid, 500 μL Tris–HCl buffer (20 mM, pH 7) and 500 μL 2 mM linoleic acid solution were incubated with 2 μL enzyme solution (22 μg purified enzyme) for 30 min at 22 °C on an orbital shaker. Reaction products were compared to those obtained with LOX-1 from soybean using the same conditions at pH 10 (borate buffer, 100 mM).

2.7. Substrate specificity

Activity of the recombinant enzyme toward trilinolein and methyl linoleate was analyzed by the photometrical assay described above or LC–MS methods, respectively. The substrate solution was prepared as described, without addition of NaOH.

2.8. Preparation of racemic hydroperoxy linoleic acid

Fenton's reaction was performed to obtain racemic hydroperoxides of linoleic acid as reference compounds. Aqueous solutions of hydrogen peroxide (9.88 M), $FeCl_2$ (25 mM) and linoleic acid (25 mM with 8 mM Tween 20) were mixed in a 5 mL volumetric flask and filled up with distilled water to reach final concentrations of 100 mM, 0.1 mM and 8.25 mM, respectively. The mixture was incubated for 20 h at 22 °C in the dark.

2.9. Synthesis of hydroxy linoleic acid

The oxidation products obtained enzymatically or by Fenton's reaction were acidified by addition of 1% formic acid and extracted using 3× 1.5 mL and 3× 5 mL *n*-hexane, respectively. Combined extracts were washed with sodium chloride solution and dried over Na_2SO_4. Solvent was evaporated under a nitrogen stream and residue was resolved in 100 μL or 2 mL methanol, respectively. To reduce the hydroperoxides, $NaBH_4$ was added and samples were incubated for 30 min at 0 °C. Subsequently, the mixture was acidified with 1% formic acid and extracted with 3× 1 mL and 3× 5 mL *n*-hexane, respectively. After evaporation of the solvent, enzymatic