All three acyl moieties of trilinolein are efficiently oxygenated by recombinant His-tagged lipid body lipoxygenase in vitro

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Abstract Recently, we found a 13-lipoxygenase in germinating cucumber cotyledons, which was located at the lipid body membrane. Based on its products formed mobilization of storage lipids seems to be initiated by this 13-lipoxygenase. For biochemical characterization its cDNA was expressed as His-tagged protein. Active recombinant enzyme was obtained from low temperature cultivation of *E. coli* after affinity purification. It (i) exhibited an unchanged region specificity, and (ii) showed a pH optimum of 7.2 against trilinolein as substrate. We compared its ability to oxygenate trilinolein with the one of another 13-lipoxygenase, soybean lipoxygenase-1. At the pH optimum of soybean lipoxygenase-1 (9.0), trilinolein was oxygenated only to 28% of the amount converted by the lipid body lipoxygenase. Moreover, trilinolein oxygenation by soybean lipoxygenase-1 leads mainly to monohydroperoxy derivatives, whereas oxygenation by lipid body LOX leads to a trihydroperoxy derivative.

Key words: Cucumber; Oxygenation of trilinolein; Soybean lipoxygenase; Substrate specificity

1. Introduction

Lipoxygenases (linoleate:oxygen oxidoreductase; EC 1.13.11.12; LOXs) occur ubiquitously in plants and animals [1,2]. They are non-heme iron containing dioxygenases which catalyze the region- and stereoselective oxygenation of polyenic fatty acids leading to the corresponding hydroperoxy derivatives [3]. Plant LOXs may be classified into 9- and 13-LOXs with respect to their positional specificity in linoleic acid oxygenation. Recently, a more comprehensive classification of plant LOXs has been proposed based on the comparison of their primary structures [4]. Although plant LOXs have been extensively characterized with respect to their protein-chemical and enzymatic properties there is no general concept for their biological function [3].

While data on possible functions of 9-LOXs in plants are still scarce, several functions for 13-LOXs have been suggested over the last years: (i) They have been implicated in the biosynthesis of jasmonic acid [5]. An important and probably essential role of this phytohormone seems to be its operation as a 'master switch', in signal transduction pathways of plants responses to predation and pathogen attack [6]. (ii) For many years, plant LOXs have been considered to oxygenate mainly free polyenic fatty acids forming oxygenated derivatives which may exhibit biological activities [3]. However, more recent studies suggested that certain plant LOXs are able to oxygenate not only free polyenic fatty acids but also ester lipid substrates, such as phospholipids, or even biomembranes [7,8]. Although these experiments indicate the ability of certain LOXs to accept complex ester lipids as substrates, it was unclear whether such reactions actually occur in vivo. Recently, we found that a special linoleate 13-LOX is synthesized at very early stages of germination of cucumber seedlings, is translocated to the lipid bodies and oxygenates the storage triacylglycerols [9]. Moreover, we found large amounts of oxygenated free linoleic acid derivatives in the cytosol of cotyledons suggesting that these derivatives are preferentially cleaved from the lipid stores [10]. Although the enzyme responsible for this release has not been characterized, these data suggest that the mobilization of storage lipids for β-oxidation is initiated by this linoleate 13-LOX but not by a lipase [11,12].

In order to characterize the substrate specificity of this lipid body LOX against polyenic fatty acids within triacylglycerols, we cloned a cDNA coding for the 13-LOX of lipid bodies into the pQE-30 expression vector. In particular, the N-terminal part was fused to a His-tag for purification by affinity chromatography. We compared the affinity of purified recombinant lipid body LOX with soybean LOX-1 in their ability to oxygenate trilinolein (TL).

2. Materials and methods

Lipid body LOX cDNA (LOXpSport-1) [13] was cloned in two steps into the pQE-30 expression vector. At first a 5′-part of LOXpSport-1 was amplified by PCR. The antisense primer was specific for the *Sac*I site within the coding region and the sense primer introduced a *Bam*HI site in front of the start codon. This PCR fragment was cloned into the pQE-30 expression vector and was designated as N-term.LOXpQE-30. Then the *Sac*I/*Aat*II fragment of LOXpSport-1 was ligated into the *Sac*I/*Sma*I site of N-term.LOXpQE-30 giving LOXpQE-30. LOXpQE-30 was used to transform *E. coli* M15 (pRep4). Recombinants were grown overnight at 37°C in 20 ml LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Four ml of this culture were added to 200 ml of the same medium. After growth at 37°C up to a cell density of A_{600}=0.7, the culture was supplemented with 2 mM IPTG at 8°C for 16 h. Cells of 1 l culture were collected, suspended in 40 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, containing 10% (v/v) glycerol, 0.1% Tween-20, 0.5 M NaCl), and then disrupted using a sonifier tip with five pulses each of 30 s according to [14]. The cellular debris was removed by centrifugation (12 000×*g* for 15 min). The supernatant was applied onto 0.5 ml of a Ni-chelate agarose column (Qiagen, Germany) equilibrated with lysis buffer. Unbound protein was washed with 5 ml of lysis buffer, pH 6.3. The recombinant protein was eluted with 2 ml of 50 mM Tris-HCl, pH 3.6, containing 10% (v/v) glycerol, 0.1% Tween-20, 0.5 M NaCl, and 100 mM EDTA.

LOX activity was measured polarographically by determining oxygen uptake using a Clark-type electrode in the appropriate buffers (see below) at 25°C. Oxygenation of linoleic acid or trilinolein was carried out by incubating 18 U of the affinity-purified recombinant lipid body LOX or LOX-1 from soybean (Sigma, Germany), respectively, with the substrate (120 μM final concentration) in 0.2 M potassium phos-

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phate buffer, pH 7.2 (lipid body LOX), or 0.2 M sodium borate buffer, pH 9.0 (soybean LOX-1), respectively, for 45 min at room temperature. The substrate was prepared as described before [15]. The reaction products were extracted with three volumes of a chloroform/methanol mixture (2:1, by vol.). After recovery of the organic phase, solvents were evaporated by vacuo and the lipids were reconstituted in 0.1 ml of HPLC solvent. HPLC analysis of the free fatty acid derivatives and of oxygenated trilinolein was carried out as described before [9].

3. Results

3.1. Biochemical properties of the recombinant lipid body LOX

In order to estimate biochemical properties of the lipid body LOX from cucumber cotyledons, we performed bacterial expression and purification of the recombinant protein by affinity chromatography. *E. coli* cells, containing LOXpQE-30 and grown at 37°C for 3 h, expressed the His-tagged protein as approximately 5% of the total protein as estimated by SDS-PAGE and immunoblotting (data not shown). However, no LOX activity was detectable upon affinity chromatography of the recombinant enzyme. In accordance with earlier reports active enzyme with an amount of 4 to 5 mg/l culture medium was obtained after affinity chromatography, when cells were grown at 8°C for 16 h [14]. Under these conditions the recombinant protein was expressed as 1% of the total protein.

To characterize the biochemical properties of the His-tagged protein, we compared its product specificity against linoleic acid with the purified LOX obtained from lipid bodies of 4-day-old cucumber cotyledons [16]. Both enzymes showed identical region specificity of the products (Table 1). The unusual region specificity against arachidonic acid which is in favor of (8*S*,5*Z*,9*E*,11*Z*,14*Z*)-hydroperoxy-(5,9,11,14)-eicosatetraenoic acid was also found with the recombinant enzyme (data not shown) [17].

The pH optimum of the purified enzyme from cucumber cotyledons has been determined earlier for free linoleic acid to be rather narrow at pH 8.5 [16]. To characterize this enzyme against one of its physiological substrates, we tested the pH optimum of the recombinant enzyme for TL [9]. The results are shown in Fig. 1. In contrast to the sharp pH optimum obtained for free linoleic acid, the activity optimum of lipid body LOX against TL was shifted towards a more neutral pH around 7.2.

3.2. Contrasting from the soybean LOX-1, the recombinant lipid body LOX oxygenates TL completely

Studies using polyenic fatty acids esterified within phospholipids or triacylglycerols as substrates demonstrated that preferentially plant 13-LOXs oxygenate these substrates [7–9,15,18]. Here, we ask the question whether there exist differences in the degree of oxygenation of triacylglycerols between different 13-LOXs. Therefore, we compared recombinant lipid

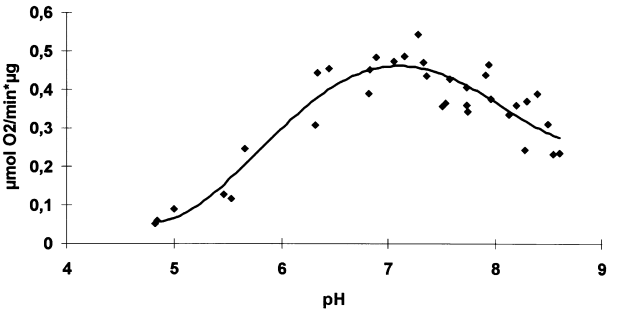

Fig. 1. Analysis of the pH optimum of recombinant lipid body-LOX acting on TL. LOX activity was measured polarographically, by determining the oxygen uptake using a Clark-type electrode.

body LOX and soybean LOX-1 in oxygenating TL. To quantitate these differences, the amount of oxygenated TL formed under in vitro conditions was estimated. When the amount of oxygenation was compared at the pH optimum of soybean LOX-1 (pH 9.0), it was only 28% of that obtained with lipid body LOX (Table 2), indicating a higher capacity of lipid body LOX to oxygenate TL. Moreover, the oxygenated products formed by both enzymes were different (Fig. 2). The soybean LOX-1 oxygenated mainly the linoleoyl moiety in position 1 or 3 of the triacylglycerol backbone leading to a monohydroperoxy derivative of TL. The lipid body LOX, however, was able to oxygenate TL in all three positions leading to almost equal amounts of monohydroperoxy and trihydroperoxy derivatives of TL (Fig. 2A vs. B, Table 2). Under reaction conditions optimized for lipid body LOX, i.e. at pH 7.2, this enzyme was capable of oxygenating TL mainly to the trihydroperoxy derivative of TL (Fig. 2C; Table 2, bottom line).

4. Discussion

Here we report for the first time the expression of an enzymatically active LOX containing an N-terminal His-tag. High level expression of foreign cDNAs coding for enzymes in bacteria has often resulted in the production of inactive expression products which are found in inclusion bodies. In this work, extremely low temperature incubation (at 8°C) of the transformed cells may prevent the formation of inclusion bodies and resulted in a high yield of the active enzyme in a soluble form. Similar temperature dependence has been reported for the expression of several other LOXs at 16°C [14,19,20]. However, a major disadvantage of the work reported before was the purification of the protein product by time-consuming chromatographic steps [21,22]. The use of an affinity tag fused to the N terminus led to a protein with additional 11 amino acid residues at the N terminus. However, this procedure did not alter the positional specificity of

Table 1
Analysis of the regio specificity of the products from the reaction of lipid body LOX purified from cucumber cotyledons or *E. coli*, respectively, with linoleic acid

Enzyme source	Positional isomers with LA		
	13-(9*Z*,11*E*)-HODE (R:S)	:	9-(10*E*,12*Z*)-HODE (R:S)
Lipid body LOX from cucumber cotyledons	87 (3:97)	:	13 (10:90)
Lipid body LOX after bacterial expression as His-tagged protein	84 (3:97)	:	16 (11:89)

Oxygenated fatty acid derivatives were isolated by RP-HPLC. Positional isomers of hydroxy linoleic acid (HODE) were given as molar ratios as determined by SP-HPLC. Molar ratios of S and R optical isomers were determined by CP-HPLC.

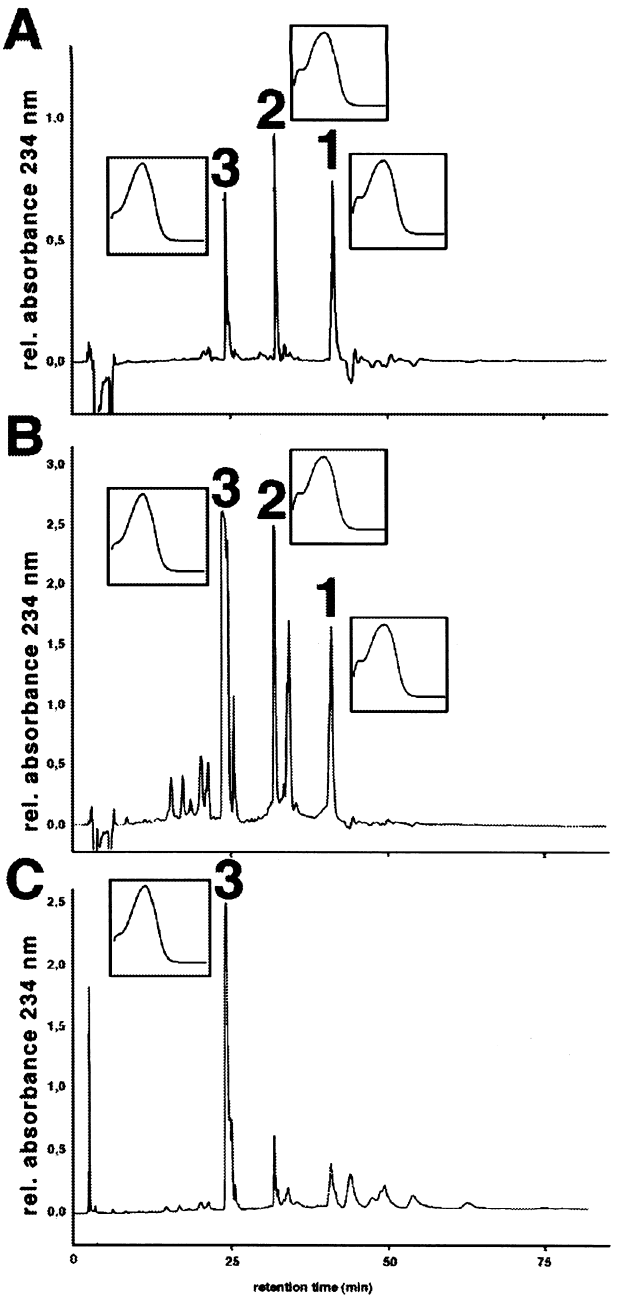

Fig. 2. HPLC analysis of esterified products obtained from the reaction of soybean LOX-1 and recombinant cucumber lipid body LOX with TL. LOXs were incubated with an emulsion of TL for 45 min at room temperature. The remaining lipids were extracted and analyzed by RP-HPLC as described [15]. A: Soybean LOX-1; B, C: recombinant lipid body LOX. All experiments were performed with the same activities of 18 U for both enzymes. Experiments A and B were performed at pH 9.0, the pH optimum of soybean LOX-1. Experiment C was performed at pH 7.2, the pH optimum of lipid body LOX. The amounts of injected lipids were the same for all traces. The numbers mark the resulting LOX-derived products: 1 indicates the monohydroperoxy derivative of TL, 2 indicates the dihydroperoxy derivative of TL, and 3 indicates the trihydroperoxy derivative of TL. All substances were characterized by coelution of authentic standards and by UV spectra with a maximum at 234 nm (maximum for conjugated diene system, insets).
←

the His-tagged protein (Table 1). Therefore, the resulting affinity-purified protein turned out to be useful for investigations of the substrate specificity and might be further used for analyzing the enzyme by site-directed mutagenesis.

Recently, the substrate specificity of a linoleate 9-LOX and linoleate 13-LOX from germinating barley embryos has been compared [15]. It was found that both LOX forms were capable of oxygenating esterified polyenic fatty acids. However, by comparing the K_m values for free polyenic fatty acids vs. esterified polyenic fatty acids it has been shown that a linoleate 9-LOX preferentially oxygenates free fatty acids whereas a linoleate 13-LOX was capable of oxygenating TL to significantly higher degrees in all positions of the triacylglycerol backbone. These findings are in accordance with an active site model of LOXs as suggested before [23,24]. For 9-LOXs, the carboxylate anion of the substrate may be the binding or recognition site within the catalytic pocket of the enzyme, whereas in the case of 13-LOXs the unpolar hydrophobic tail of the fatty acid may be orientated towards the catalytic pocket of the enzyme. This may explain why unpolar substrates, such as TL, are preferred substrates for linoleate 13-LOXs. In this report we asked the question whether there are differences between different linoleate 13-LOXs with respect to their capability of oxygenating complex substrates such as TL. Lipid body LOX oxygenates TL (i) in higher amounts compared to soybean LOX-1, and (ii) to a higher degree as shown by 76% vs. 12% of the trihydroperoxy derivative of TL (Fig. 2 and Table 2). This might be due to different physiological functions of different linoleate 13-LOXs as has been suggested before [7,11]. One may expect that different sets of 13-LOXs exist. One group involved in oxygenating mainly free fatty acids leading to their degradation in the β-oxidation during normal lipid turnover [25] and another set of 13-LOXs was capable of oxygenating esterified polyenic fatty acids responsible for changes in the membrane structure or the degradation of storage lipids [12].

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Table 2
Analysis of the products from the reaction of soybean LOX-1 or lipid body LOX with TL

Enzyme source	Amount of TL oxygenation	Molar ratios of oxygenation products of TL				
		TL-3-HODE	:	TL-2-HODE	:	TL-1-HODE
Soybean LOX-1, pH 9.0 (A)	28%	12	:	19	:	69
Lipid body LOX, pH 9.0 (B)	100%	36	:	18	:	46
Lipid body LOX, pH 7.2 (C)	–	76	:	9	:	15

Oxygenated fatty acid derivatives were analyzed by RP-HPLC as described before [9] and shown in Fig. 2. Molar ratios were given for all oxygenated derivatives of TL.

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