The lipid body lipoxygenase from cucumber seedlings exhibits unusual reaction specificity

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Abstract The lipid body lipoxygenase of cucumber seedlings is at a high level expressed during the germinating process which is the stage of triglyceride mobilisation. This enzyme exhibits an unusual positional specificity which has not been described so far for any plant and animal lipoxygenase. The purified enzyme converts arachidonic acid to 15-S-hydroperoxy-5Z,8Z,11Z, 13E-eicosatetraenoic acid (15S-HPETE), 12-S-hydroperoxy-5Z, 8Z, 10E, 14Z-eicosatetraenoic acid (12S-HPETE), and 8-S-hydroperoxy-5Z,9E,11Z,14Z-eicosatetraenoic acid (8S-HPETE) in a ratio of 76:4:20 with the corresponding R-isomers being only minor contaminants. Binding to the lipid bodies enhances the arachidonic acid dioxygenase activity more than 4-times and alters positional specificity of the enzyme in favour of 8-S-hydroperoxy-5Z,9E,11Z,14Z-eicosatetraenoic acid (8S-HPETE) formation.

Key words: Cucumis sativus; Lipid body; Lipoxygenase; Positional specificity; Membrane binding

1. Introduction

Lipoxygenases (linoleate:oxygen oxidoreductase; EC 1.13.11.12) are widely distributed in plants and animals [1,2]. They catalyse the regio- and stereo-specific oxygenation of polyunsaturated fatty acids to their corresponding hydroperoxy derivatives [3]. The mechanism of the lipoxygenase reaction involves a stereoselective hydrogen removal form a doubly allylic methylene and a subsequent stereo-specific insertion of molecular oxygen [4]. Since linoleic acid does only contain one doubly allylic methylene (C-11), hydrogen can only be removed from this carbon atom. Consequently, only two positional isomers, namely 13- and 9-hydroperoxy-octadecadienoates, may be formed. In contrast, arachidonic acid contains 3 doubly allylic methylenes and thus 6 positional isomers of hydroperoxy eicosanoid isomers may be formed. In the past lipoxygenases have been classified according to their positional specificity of arachidonic acid oxygenation [2]. For a long time lipoxygenases were believed to exhibit a singular positional specificity. However, more recent data indicated that certain enzymes exhibit a dual or even multiple positional specificity [5–8].

Recently we described a lipoxygenase which is synthesised in the cotyledon of cucumbers during the early germination process. This enzyme is found at the lipid bodies during the stage of lipid mobilisation of the germinating process [9]. This lipoxygenase was isolated as homogenous enzyme from the lipid bodies and can be distinguished from other isoforms by size, by charge and marked differences in pH-optimum [10,11]. So far the biological function of this lipoxygenase is not clear. However, the fact that it is specifically synthesised during the stage of lipid mobilisation during the germinating process suggests that it may be functionally related to this process [9]. The reaction specificity of the cucumber lipid body lipoxygenase has not been studied in detail. Here we report that this enzyme exhibits an unusual multiple positional specificity of arachidonic acid oxygenation and that its oxygenase activity is enhanced after binding to the lipid bodies.

2. Materials and methods

Dry seeds of cucumber (*Cucumis sativus* L.) or cotyledons of one-day-old cucumber seedlings were used for enzyme preparation. Lipid body fractions and solubilized lipoxygenase of lipid bodies were prepared according to [10]. For product analysis the enzyme was incubated with 300 μM arachidonic acid for 15 min in 50 mM sodium borate buffer, pH 8.0. Reactions were stopped by the addition of sodium borohydride which reduces the hydroperoxy fatty acids formed to their corresponding hydroxy compounds. The mixture was acidified to pH 3 with glacial acetic acid. The lipophilic products were twice extracted with 1 ml of ethyl acetate. The organic extracts were combined, the solvent was evaporated and the remaining lipids were redissolved in 100 μl of methanol.

The hydroxy fatty acids were isolated from the product mixture by reverse phase high performance liquid chromatography (RP-HPLC) and the positional isomers were separated by straight-phase HPLC (SP-HPLC). The enantiomer composition of the positional isomers was determined by chiral phase high-pressure liquid chromatography (CP-HPLC) according to [18].

3. Results

3.1. Product specificity of cucumber lipid body lipoxygenase

During the process of germination a lipoxygenase form of cucumber seedlings is associated with the lipid bodies as indicated by immunocytochemical studies [11]. To obtain information about the positional specificity of this lipoxygenase the lipid bodies were isolated and incubated with radioactivity labelled arachidonic acid. SP-HPLC analysis of the hydro-(pero)xy eicosanoids formed indicated the formation of 3 major radioactively labelled isomers (Fig. 1). 8-S-hydroperoxy-5Z,9E, 11Z,14Z-eicosatetraenoic acid (8-HETE) was detected as main product (63%), followed by 15-S-hydroperoxy-5Z,8Z,11Z, 13E-eicosatetraenoic acid (15-HETE; 25%), and 12-S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE; 12%). The chemical structures of the products were confirmed by UV-spectroscopy and GC-MS analysis (data now shown). In addition, another compound absorbing at 234 nm was detected which co-chromatographed with an authentic standard of 13-hydroxy-9Z,11E-octadecadienoic acid. Since this product was not radioactively labelled it must originate from endogenous

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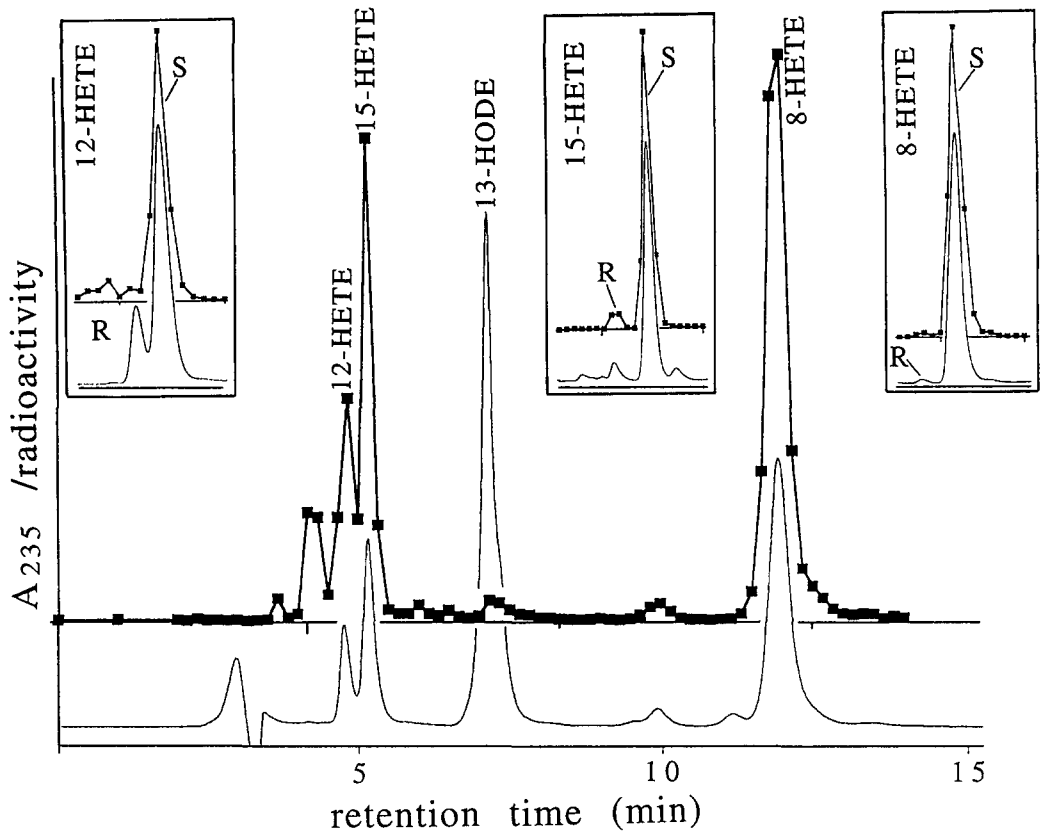

Fig. 1. HPLC analysis of the arachidonic acid oxygenation products. Lipid bodies which contain the lipoxygenase as major protein were prepared from one-day-old cucumber cotyledons and incubated with [1-^{14}C]arachidonic acid (185 kBq/ml), what corresponds to an arachidonic acid concentration of 100 μM in 0.1 M phosphate buffer, pH 8.0, for 15 min. After reduction with sodium borohydride the resulting hydroxy fatty acids were extracted, prepared on RP-HPLC, and analysed on SP-HPLC on a Nucleosil 50–7 column (Macherey-Nagel, Germany) with a solvent system consisting of hexane/2-propanol/acetic acid (100:2:0.1; by vol.) and a flow rate of 1 ml/min. The absorbance at 234 nm (solid line) was monitored. The radioactivity (dotted line) was determined by measuring it in each fraction in a liquid counter. Insets: chiral phase HPLC of the positional isomers separated. Analysis were carried out on a Chiralcel OD column (Baker Inc., USA) with a solvent system consisting of hexane/2-propanol/acetic acid (100:2:0.1; by vol.) and a flow rate of 1.5 ml/min. The 2-propanol concentration was varied as follows: 12-HETE 2%, 8-HETE 3% and 15-HETE 5%.

lipids of the lipid bodies. Remarkably, no 5-S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE) was formed by the lipid body lipoxygenase. Analysis of the enantiomer composition of the major oxygenation products indicated an almost exclusive formation of the S-isomer for 8-, 15, and 12-HETE (insets to Fig. 1).

3.2. *Binding to the lipid bodies activates the lipoxygenase and altersits positional specificity*

For the reticulocyte lipoxygenase it has been shown that the enzyme is activated when binding to biomembranes [12]. In order to find out whether the cucumber seed lipoxygenase is activated when transferring to the target lipid organelles, the activity of the purified enzyme was assayed before and after binding to lipid bodies of dry cucumber seeds. As indicated before, lipid bodies form dry seeds did not contain the lipoxygenase [11]. Comparing the total amounts of HETE's formed by the purified and the membrane bound enzyme (3.3 μg vs. 16 μg) showed that the lipid body lipoxygenase is activated more than 4-fold after binding to the lipid bodies.

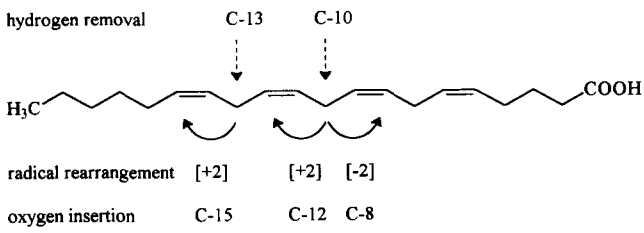

Fig. 2. Factors influencing the positional specificity of lipoxygenases.

Comparison of the product pattern formed in vivo by lipid body lipoxygenase and in vitro before and after binding to the lipid storage organelles indicated changes in positional specificity of the enzyme (Table 1). Binding to the lipid bodies increased the formation of 8- and 12-H(P)ETE both of which originate from a C-10 hydrogen removal, approaching the product pattern obtained in the in vivo situation (Fig. 1). The percentage of 15-HETE which involves a C-13 hydrogen removal was decreased. In contrast, the product pattern formed by the reticulocyte lipoxygenase did not change after incubation with the lipid bodies (data not shown).

4. Discussion

Lipid bodies are formed during seed maturation [13,14] and receive a new set of proteins during onset of germination, i.e. at the beginning of their degradation [15,16]. The most highly

Table 1
Alteration of the positional specificity after binding to the lipid bodies

Lipoxygenase preparation	Product composition (%)			12-HETE + 8-HETE/15-HETE ratio
	12-HETE	15-HETE	8-HETE	
Solubilized enzyme	4	76	20	0.3
Solubilized enzyme + lipid bodies from dry seeds (in vitro recombination)	10	57	33	0.75
Lipid bodies from 1-day-old seedlings (in vivo recombination)	12	25	63	3

Incubation, product preparation and analyses as described in the legends to Figs. 1 and 2. Quantification of the product pattern was carried out by SP-HPLC. The 12-HETE + 8-HETE/15-HETE ratio represents the relation of C-10/C-13 hydrogen removal during arachidonic acid oxygenation.

expressed protein was identified as a lipoxygenase [9], which could be distinguished from other lipoxygenase isoforms by its size, by its charge, by differences in the pH-optimum, and its temporal appearance during germination [10,11]. We hypothesised that the enzyme might contribute to make the stored lipids available as carbon source for the germinating process [11].

For a long time lipoxygenases have been believed to exhibit a singular positional specificity. The positional specificity of a lipoxygenase is determined by two processes [3,4]: (i) position of the hydrogen removal; and (ii) position of the oxygen insertion (Fig. 2). In principle, hydrogen removal from one double allylic methylene allows a [−2] or a [+ 2] radical rearrangement and consequently a [−2] or a [+ 2] oxygen insertion [4]. Potato lipoxygenases [6,7] and the tomato fruit lipoxygenase [8] are capable of catalysing hydrogen abstraction from different doubly allylic methylenes of arachidonic acid but always catalyses a [−2] oxygen insertion. On the other hand, the soybean lipoxygenase-2 and the pea lipoxygenase-1 catalyse the formation of 9- and 13-HPODE from linoleic acid which involves a [−2] and [+ 2] radical rearrangement. In both cases the positional isomers were shown to be racemic mixtures [17,18]. In contrast, the cucumber lipid body lipoxygenase investigated here catalyses the formation of three chiral oxygenation products from arachidonic acid. Two of which originate from C-10 hydrogen removal and involve a [+ 2] and [−2] radical rearrangement (12- and 8-HETE, respectively). The formation of 15-HPETE involves a C-13 hydrogen removal. It is remarkable that neither 11-HETE which would be formed via [−2] radical rearrangement following a C-13 hydrogen removal nor 9- and 5-HETE both of which originating from a C-7 hydrogen abstraction could be detected. According to our knowledge this is the first report on a lipoxygenase which is capable of catalysing the simultaneous formation of chiral oxygenation products which involve a [−2] and a [+ 2] radical rearrangement (8S-HETE vs. 12S- and 15S-HETE). It has been reported before that the specificity of lipoxygenases may be altered in vitro by variation of the pH [19] and changes in the structure of the substrate [20]. The observation that the positional specificity of the cucumber lipid body lipoxygenase is altered after binding to the lipid storage organelles indicates that lipoxygenase specificity may be altered during membrane binding. It is well known that mammalian lipoxygenases are capable of translocating to membranes [21,22,23]. For the 5-lipoxygenase a special docking protein called FLAP has been identified [24]. No obvious changes in the positional specificity of the human 5- and the rabbit 15-lipoxygenase has been detected after membrane binding. However, it remains to be investigated whether the specificity of other mammalian and plant lipoxygenases is changed during membrane binding and whether such an alteration will influence the biological activity of the products formed.

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