Comparison of the Substrate Specificities of Lipoxygenases Purified from Soybean Seed, Wheat Seed, and Cucumber Cotyledons

Kenji Matsui, Hiroyuki Shinta, Hiromitsu Toyota, Tadahiko Kajiwara, and Akikazu Hatanaka

Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan

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Lipoxygenases were highly purified from soybean seed, wheat seed and cucumber cotyledons. Substrate specificities of these lipoxygenases were studied by using an entire series of ($\omega 6Z,\omega 9Z$)-C_{13}~C_{24}-dienoic acids as synthetic substrate analogues. Soybean lipoxygenase-1 and cucumber lipoxygenase showed broad specificities for these substrates while wheat lipoxygenase showed narrow specificities. Position of dioxygenation to each substrate was analyzed by high performance liquid chromatography. With soybean lipoxygenase-1 elongation of the distance between the terminal carboxyl group and the site of hydrogen removal in a substrate decreased the positional specificity of dioxygenation, while, with cucumber lipoxygenase, shortening the distance decreased the specificity. It was suggested that cucumber lipoxygenase and soybean lipoxygenase-1 recognized the terminal carboxyl group of a substrate to arrange it only in one orientation at the reaction center. In case of wheat lipoxygenase, recognition of the carboxyl group was thought to have crucial and essential role to secure the activity.

Introduction

Lipoxygenase (EC 1.13.11.12) catalyzes the stereospecific dioxygenation of unsaturated fatty acids containing at least one methylene-interrupted (*Z,Z*)-pentadiene system. Lipoxygenases in plants can be classified into two types which are distinguishable primarily by differences in their pH-activity profiles. Type 1 enzymes show optimum activity around pH 9 while type 2 enzymes are most active between pH 6.5 and 7 [1]. Soybean lipoxygenase-1 is a well characterized type 1-lipoxygenase. Plant lipoxygenases can be also classified by their product specificities. Soybean lipoxygenase-1 and cucumber cotyledons lipoxygenase oxygenize linoleic acid to form (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid while removing a pro-(*S*) hydrogen from the bis allylic methylene. Whereas linoleic acid 9-lipoxygenases such as those in wheat seed and potato tuber catalyze the formation of (9*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid while removing a pro-(*R*) hydrogen from the bis-allylic methylene [2]. Because pentadiene system of a substrate is symmetrical, hydrogen removal and O_2 insertion are carried out by both the types of lipoxygenase in spatially identical manner. Thus, strict positional specificities of dioxygenation observed with most lipoxygenases indicate that there exists a mechanism to recognize the orientation of a fatty acid. Gardner [3] reported that soybean lipoxygenase-1 recognizes a carboxylate anion to arrange a fatty acid only in one orientation.

In our previous paper [4], we have examined substrate and product specificity of soybean lipoxygenase-1 using an entire series of ($\omega 6Z,\omega 9Z$)-C_{13}~C_{20}-dienoic acids, which have different distances from the terminal carboxyl group to the pentadiene moiety, and suggested that the interaction of a carboxylate anion of a substrate with the reaction center of soybean lipoxygenase-1 was important to oxygenize a specific position of a substrate but hydrophobic interaction and recognition of the pentadiene system were also important to secure the activity.

It seemed of interest to try to reveal whether the manner of substrate recognition postulated for soybean lipoxygenase-1 can be generalized to those of other plant lipoxygenases. To this end, we purified lipoxygenases not only from soybean seed, but also from wheat seed and cucumber cotyledons which differ in optimum pHs and/or product specificities. Substrate and product specificities of these three lipoxygenases were compared by using an entire series of ($\omega 6Z,\omega 9Z$)-C_{13}~C_{24}-dienoic acid.

Reprint requests to Kenji Matsui.

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Materials and Methods

Purification of lipoxygenases

Soybean lipoxygenase-1 was purified from soybean seed (*Glycine max* L. *cv.* Tamahomare) as described [5]. The other isozymes were carefully removed. In a typical experiment soybean lipoxygenase-1 was purified 20-fold with a specific activity of 134.5 U/mg protein.

Cucumber lipoxygenase was purified from cotyledons of cucumber seedlings (*Cucumis sativus* L. *vc.* Suyo). The cotyledons (6 day old) were homogenized with 2 volumes of 20 mM sodium phosphate buffer, pH 8.0, containing 5% polyclar-AT, 1 mM EDTA, 0.5 mM phenylmethanesulfonylfluoride and 1 mM sodium ascorbate. The filtrate through 8 layers of cheese cloth was centrifuged at 80,000 × *g* for 80 min and the resultant supernatant was fractionated with ammonium sulfate (20–40% saturation). After dialysis, the fractionated solution was successively separated with a DEAE-Cellulofine A-500 (Seikagaku Kohgyo Co. Tokyo) column and a Butyl-Toyopearl 650 M (Tosoh, Tokyo) column. In a typical experiment cucumber lipoxygenase was purified 19-fold with a specific activity of 103.0 U/mg protein.

Wheat lipoxygenase was purified from wheat seed (*Triticum vulgare* Desf. *cv.* Nourin 61 gou). Wheat seed was finely ground and the flour was stirred with three volumes of 0.12 M sodium phosphate buffer, pH 6.9, at 4 °C for 1 h. After filtrated through 4 layers of cheese cloth, the filtrate was centrifuged at 23,000 × *g* for 20 min and the supernatant was fractionated with ammonium sulfate (35–55% saturation). After dialysis against 10 mM sodium phosphate buffer, pH 6.9, the solution was applied to a DEAE-Cellulofine A-500 column (20 mm × 305 mm) equilibrated with the same buffer. On this step three peaks of lipoxygenase activities appeared and the most active fraction which was eluted fastest was collected. The active fraction was concentrated by ultrafiltration with Ultra Filter UP-20 (Advantec Toyo, Tokyo) and was dialyzed against 10 mM Tris-Cl buffer, pH 8.0. The dialysate was applied to a QA-824 column (Showa Denko, Tokyo, 8 mm × 75 mm) equilibrated with the same buffer, and eluted with a linear gradient of NaCl concentration (0–0.1 M) formed with the same buffer. The active fraction was concentrated with Centricon-30 (Amicon) and was applied to a TSK-gel G-3000SW column (Tosoh, Tokyo, 7.5 mm × 600 mm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1 M sodium sulfate. The active fraction was eluted with the same buffer and used as purified wheat lipoxygenase. In a typical experiment wheat lipoxygenase was purified 450-fold with a specific activity of 155.4 U/mg protein.

Enzyme assay

Linoleic acid (99% pure) was purchased from Sigma Chemical Co. ($\omega 6Z,\omega 9Z$)-C_{13}~C_{20}-dienoic acids were prepared as previously described [4]. ($\omega 6Z,\omega 9Z$)-C_{21}~C_{24}-dienoic acids were synthesized *via* the Wittig coupling between a C_9-phosphorane salt and C_{11}~C_{14}-aldehydes as in [5]. Geometrical purity of the synthesized fatty acids were determined with HPLC analyses as previously described [6]. All the dienoic fatty acids were used as substrates after purificated by silica gel column chromatography to 99% purity.

Lipoxygenase activities were determined spectrophotometrically at 25 °C by following the formation of hydroperoxides at 234 nm (ε = 25,000 $M^{-1} \cdot cm^{-1}$). The buffers used were, for soybean lipoxygenase-1; 50 mM sodium borate buffer (pH 9.0), for cucumber lipoxygenase; 50 mM sodium phosphate buffer (pH 6.3) and for wheat lipoxygenase; 50 mM sodium phosphate buffer (pH 6.9). To 0.98 ml of the respective buffer, 10 µl of substrate solution (10 mM dienoic acid dispersed with 0.2% Tween 20) was added and the reaction was started by the addition of 10 µl of enzyme solution (7.0×10^{-3} U). One unit of enzyme activity was expressed as the amount of enzyme forming 1 µmol of hydroperoxides at 25 °C.

Product specificity

Each lipoxygenase (1 U) was added to a reaction mixture consisting of 50 ml of the respective buffer containing 1 ml of the substrate solution and incubated for 10 h at 4 °C under O_2 atmosphere. Then the reaction mixture was acidified with 2N HCl and extracted with 100 ml of ether. The ether layer was washed twice with 100 ml of saturated NaCl solution, and was evaporated *in vacuo*. Then 3 ml of ether was added to the residue and the ether solution was treated with ethereal diazomethane and subsequent triphenylphosphine to give the corresponding hydroxy-ester. Compositions of posi-

tional isomers of the products were analyzed by straight phase HPLC (Shimadzu LC-5A) equipped with a Zorbax-SIL column (Dupont-Shimadzu, 4.6 mm × 250 mm) with detection at 234 nm. Elution was carried out with *n*-hexane/*iso*-propanol (99/1, v/v) at a flow rate of 1 ml/min at 25 °C.

Results and Discussion

Lipoxygenases purified from soybean seed, wheat seed and cucumber cotyledons were almost homogenous when analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Reactivities of the substrates, ($\omega 6Z,\omega 9Z$)-C_{13}~C_{24}-dienoic acids, were examined at a concentration of 100 μM. The reactions were monitored at the respective optimum pHs, *i.e.*, pH 9.0 for soybean lipoxygenase-1, pH 6.9 for wheat lipoxygenase [7] and pH 6.3 for cucumber lipoxygenase [8]. As shown in Fig. 1, the three lipoxygenases showed different profiles of substrate specificities. Soybean lipoxygenase-1 showed broad specificity having an optimum activity with C_{20}-dienoic acid. Cucumber lipoxygenase was most active with C_{19}-dienoic acid and with the longer substrate than C_{19} the activity decreased gradually like that observed with soybean lipoxygenase-1. But, contrary to soybean lipoxygenase-1, cucumber lipoxygenase little catalyzed C_{17}-dienoic acid and with C_{16}- and the shorter dienoic acids, no activity was detected. With C_{17}-dienoic acid, cucumber lipoxygenase initiated the peroxidation reaction only after a very long lag period (about 10 min) although the other lipoxygenases did not show such a long lag period even if a substrate of low reactivity was used. Furthermore, cucumber lipoxygenase did not show such a long lag period with C_{24}-dienoic acid which showed almost the same reactivity of C_{17}-dienoic acid. Lag period was believed to be the time to activate the inactive form of lipoxygenase by its own product, hydroperoxide [1, 2]. The product formed from C_{17}-dienoic acid was thought not to be an efficient activator for cucumber lipoxygenase under the reaction condition employed here.

Wheat lipoxygenase showed relatively narrow specificity when compared with the other lipoxygenases. Wheat lipoxygenase was most active with a natural substrate, linoleic acid, and either addition or deletion of only one methylene unit decreased the activity drastically. The other dienoic acids were not oxygenized by wheat lipoxygenase. Relatively narrow specificity observed with wheat lipoxygenase suggests that recognition of the terminal carboxyl function of a substrate has a crucial role. This suggestion was supported by the result obtained by Kühn *et al.* [10] who reported that the site of the hydrogen removal is determined by the distance from the carboxyl group rather than from the methyl end of the fatty acid chain.

The substrates which showed appreciable activities were oxygenated with the purified lipoxygenases under O_2 atmosphere at 4 °C and the position of dioxygenation were determined by straight-phase HPLC analyses. All the substrates used here had two possible oxygenation sites, *i.e.* ω6- and ω10-positions. As reported previously, by either

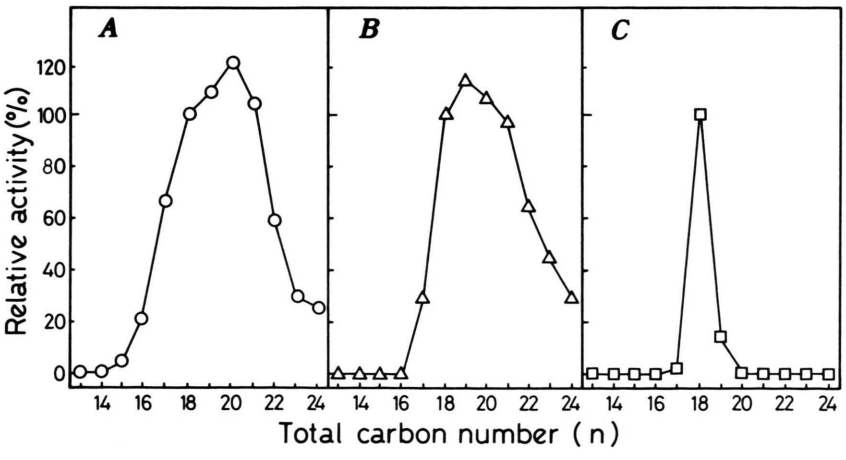

Fig. 1. Substrate specificities of soybean lipoxygenase-1 (A), cucumber lipoxygenase (B) and wheat lipoxygenase (C) for the synthetic substrates. The reactivity of a substrate relative to that of C_{18}-dienoic acid (linoleic acid) was plotted against total carbon number (*n*).

soybean lipoxygenase-1 [1] or cucumber lipoxygenase [8], linoleic acid was mainly oxygenated at ω6-position to form (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid as a main product but wheat lipoxygenase oxygenated at ω10-position to form (9*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid as a main product [10]. Soybean lipoxygenase-1 oxygenated ω6-position of a substrate shorter than C_{22} with high specificity (over 95%). But low specificities were observed with C_{23}- and, more pronouncedly, with C_{24}-dienoic acids (Table I). It must be noticed that C_{15}-dienoic acid which showed only trace reactivity was oxygenized more specifically than the two longest acids although they had higher reactivities. This indicates that a fatty acid suited for peroxidation is not always peroxidized specifically. In other words, a factor needed to be oxygenized and that needed to be specifically oxygenized are not same. Gardner [3] suggested that the carboxylate anion form of a substrate arranges itself at the active site of soybean lipoxygenase-1 only in one orientation although the carboxylic acid form of the substrate can arrange itself in either orientation in head to tail alignment. At the pH used for the assay (9.0), the carboxylate anion forms of the substrates were thought to be predominant if the result obtained with linoleic acid [9] was applicable to the other fatty acids used here. High specificity of oxygenation to the substrates shorter than C_{22} indicates that the recognition of the anion fully functions to arrange these substrates only in one orientation even if the reaction center of the enzyme is apart from the pro-(*S*) hydrogen attached to the bis-allylic methylene. On the contrary, the recognition may be weakened with the two longest dienoic acids by much elongating the distance between the anion and the site of hydrogen removal of a substrate. As a result, these substrates get more chance to arrange themselves in reversal orientation to be removed the pro-(*R*) hydrogen and oxygenized ω10-position.

Table I. Positional and geometrical specificity of plant lipoxygenases.

Total carbon number (*n*)	ω6		ω10	
	(*Z*,*E*)	(*E*,*E*)	(*E*,*Z*)	(*E*,*E*)
Soybean				
15	94.4	1.5	3.1	1.0
16	99.3	trace	0.7	trace
17	95.3	1.7	1.0	2.0
18	96.3	0.3	3.0	0.4
19	95.0	1.9	1.5	1.6
20	93.6	3.2	3.2	trace
21	90.2	5.3	2.1	2.4
22	92.6	3.4	4.0	trace
23	86.2	3.4	9.9	trace
24	75.6	2.3	22.1	trace
Cucumber				
17	68.7	4.0	19.4	7.9
18	79.2	2.9	17.4	4.1
19	72.5	4.9	16.3	6.3
20	90.0	3.4	4.4	2.2
21	92.4	4.0	2.8	0.8
22	93.1	5.2	1.1	0.6
23	90.4	7.3	1.8	0.5
24	69.2	2.5	27.6	0.7
Wheat				
17	10.8	2.8	84.1	2.4
18	7.1	2.8	87.7	2.4
19	16.1	5.3	74.7	3.9

Cucumber lipoxygenase introduced oxygen mainly to ω6-position of any substrates, which indicates that cucumber lipoxygenase recognizes the undissociated form of the terminal group to arrange a substrate only in one orientation because the carboxylic acid form of a substrate is thought to be abundant at pH 6.3. But, the distance between the terminal functional group and the site of hydrogen removal affected the positional specificity of dioxygenation of cucumber lipoxygenase to higher extent than that of soybean lipoxygenase-1. The substrates shorter than C_{19} and the longest substrate, C_{24}, were oxygenated by cucumber lipoxygenase with low positional specificities (Table I). Unexpectedly, dioxygenation to C_{19}-dienoic acid which showed the highest reactivity (see Fig. 1) was not so specific. The highest specificity was observed with C_{21}- and C_{22}-dienoic acids which showed only about 50% of the reactivity of C_{19}-dienoic acid. This result indicated that a fatty acid suited for peroxidation is not always dioxygenized specifically. The recognition of the terminal group of a substrate may not be so strong as that observed with soybean lipoxygenase-1 and the substrate recognition site of cucumber lipoxygenase can arrange a substrate shorter than C_{20} in both orientations. It seemed inconsistent that highest specificity was observed with fatty acids which were not detected in cucumber cotyledons [11]. Because cucumber cotyledons have at least two kind of hydroperoxide lyases differing in sub-

strate specificity [12], the low specificity observed with linoleic acid may have significance to explain the still-unknown physiological role of this enzyme *in vivo*.

Only little information was obtained with analyses of the products obtained with wheat lipoxygenase because only three products could be formed (Fig. 1). But, it was suggested that recognition of the carboxyl group but not carboxylate anion had an important role to arrange a substrate only in one orientation.

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