Expression of Lipoxygenase and Hydroperoxide Lyase Activities in Tomato Fruits

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The distribution (or locarization) of lipoxygenase (LOX) and hydroperoxide lyase (HPO lyase) activities in ripening and ripe tomato fruits was investigated. The highest LOX activity existed between skin and outer flesh of tomato fruits. HPO lyase showed no tissue specificity. LOX specifically formed linoleic acid 9-*E,Z*-hydroperoxide (9-*E,Z*-HPO) from linoleic acid (LA), whereas HPO lyase specifically cleaved 13-*Z,E*-HPO. Although a low level (0.36 ± 0.069 nmol/g fr.wt.) of hexanal was detected in the intact tomato fruit, HPOs were not detected. When a tomato fruit was injured by cutting it into 8 fragments and incubated at 25 °C, hexanal increased to 1.642 nmol/g fr.wt. by 30 min. By homogenizing at pH 6.3, hexanal increased to 21.1 nmol/g fr.wt. during a 30 min incubation. UV irradiation of tomato fruits also increased the formation of hexanal. From these results, LOX and HPO lyase are considered to exist as latent forms and to begin the expression of the activity upon injury.

Introduction

Lipoxygenase (EC 1.13.11.12, LOX) catalyzes the incorporation of molecular oxygen into polyunsaturated fatty acids such as linoleic acid (LA) and linolenic acid which possess the (*Z,Z*)-1,4-pentadiene structure. LOX activity has been described for many tissues in different plant species [1]. In plant tissues, fatty acid hydroperoxides (HPO) formed by the LOX reaction are further metabolized by hydroperoxide lyase (HPO lyase). HPO lyase catalyzes the cleavage of fatty acid 13-HPO and/or 9-HPO to hexanal and hexenals (C_6-aldehydes) and/or nonenals (C_9-aldehydes), respectively [2]. Kazeniac and Hall reported that tomato fruits could form C_6-aldehydes from linoleic and linolenic acids [3]. Galliard and Matthew observed that tomato fruits possessed hydroperoxide lyase activity to form C_6-aldehydes [4].

C_6-aldehydes are characteristic aroma substances of tomato fruits, but their physiological function including fatty acid hydroperoxide and 12-oxo-(9*Z*)-dodecenoic acid [5], a counterpart of the cleaved products, is unknown. Furthermore, a physiological role of LOX has not been elucidated, although the enzyme has been implicated in growth and development, senescence, wounding and pest resistance of plants [6].

As mentioned above, the existence of LOX and HPO lyase activities was reported for many plants, but little is known whether or not LOX and HPO lyase activities are expressed in intact plant tissues. Thus, expression and localization of LOX and HPO lyase activities in intact tomato fruits were studied.

Materials and Methods

Plant material and enzyme preparation

Ripening and ripe tomato fruits (*Lycopersicon esculentum* Mill., varieties unspecified) were purchased from a local supplier. Tomato fruits were separated, using a knife, into 3 parts, *i.e.*, skin containing outer flesh (stated as preparation-A, Table I), flesh without skin and locular material with seeds. In some experiments, fleshy tissue was further removed from preparation-A to obtain relatively pure skin (stated as preparation-B). Each part was cut into small pieces and homogenized with 2 vols. of 50 mM sodium acetate buffer (pH 5.5) at 5 °C [4]. After filtration through 4 layers of gauze the homogenate was used immediately as the enzyme source.

Abbreviations: 9-*E,Z*-HPO, 9-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid; 9-*E,E*-HPO, 9-hydroperoxy-(10*E*,12*E*)-octadecadienoic acid; 13-*Z,E*-HPO, 13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid; 13-*E,E*-HPO, 13-hydroperoxy-(9*E*,11*E*)-octadecadienoic acid; HPLC, high performance liquid chromatography.

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In order to examine the subcellular localization of LOX and HPO lyase, cell debris of the extract of preparation-A was removed by centrifugation at 500 × *g* for 30 sec. The resulting supernatant was further centrifuged successively at 4,000 × *g* for 10 min, at 20,000 × *g* for 20 min and at 80,000 × *g* for 80 min. Each pellet was resuspended in a small amount of homogenization buffer. Thus, crude extract, 4,000 × *g* pellet, 20,000 × *g* pellet, 80,000 × *g* pellet and 80,000 × *g* supernatant were obtained.

UV irradiation was performed with a 15 W ultraviolet fluorescence lamp (GL-15, Panasonic Co. Osaka, Japan) at 10 cm distance from the surface or the cut surface of a tomato fruit at 25 °C for 10 min.

The quantity of protein in homogenates was determined by Lowry's method with bovine serum albumin as a standard [7].

Lipoxygenase activity

LOX activity was determined by a polarographic method with a Clark-type oxygen electrode (Yellow Springs Instrument Co.). A standard reaction mixture (1.75 ml) contained 50 mM potassium phosphate buffer (pH 6.3), 0.1 ml enzyme solution and 0.05 ml substrate solution (50 mM LA in 0.2% Tween 20). One unit of enzyme was expressed as the amount of enzyme consuming 1 μmol O_2/min at 25 °C.

Preparation of linoleic acid 13- and 9-hydroperoxide

9- and 13-HPOs were prepared from LA (purified on a SiO_2 column, Wako Pure Chemicals, Osaka, 99% pure) as previously described, using LOXs from potato tuber [8] and from soybean seed (Sigma, type I) [9], respectively. 9-HPO included geometrical and positional isomers in a ratio of 9.9/8.4/81.7/0 (13-*Z,E*-HPO/13-*E,E*-HPO/9-*E,Z*-HPO/9-*E,E*-HPO). 13-HPO also included geometrical and positional isomers in a ratio of 92.8/3.2/3.2/0.8.

Geometrical and positional isomer compositions and quantity of linoleic acid hydroperoxide

Geometrical and positional isomer compositions as well as the quantity of LOX products from LA were determined from hydroxy derivatives of HPO. Potassium phosphate buffer (50 mM, 4.5 ml, pH 6.3) was poured into a 50 ml-Erlenmeyer flask and 0.1 ml of 50 mM LA in 0.2% Tween 20 (final concentration, 1 mM) was added, and the enzyme reaction was started by addition of 0.5 ml enzyme solution. After a given time of incubation at 25 °C, the reaction mixture was acidified with HCl to pH 3 and enzymatic products were extracted with *n*-hexane (5 ml, 3 times). The extracts were reduced by $NaBH_4$ under a N_2 atmosphere for 30 min at room temperature. The mixture was diluted with water, then acidified with HCl to pH 3, and extracted with *n*-hexane/diethyl ether (1/1, v/v). The extract was concentrated *in vacuo* and the concentrate was dissolved in ethanol for HPLC analysis.

HPLC analysis was performed with a Shimadzu liquid chromatograph (LC-6A) equipped with CLC-SIL (Shimadzu; 4.6 mm ID × 25 cm, carrier solvent; *n*-hexane/isopropanol/acetic acid (98.5/1/0.5, v/v) at a flow rate of 1.0 ml/min with detection at 234 nm) [10]. Hydroperoxides prepared from potato and soybean LOXs were used as standard HPOs.

Hydroperoxide lyase activity

HPO lyase and aldehydes forming activities were determined from the quantity of the 2,4-dinitrophenylhydrazone derivatives of the aldehydes formed. Potassium phosphate buffer (50 mM, 4.5 ml, pH 6.3) was poured into a 50 ml-Erlenmeyer flask and 500 mM HPO in ethanol, or 50 mM LA in 0.2% Tween 20 (at a final concentration of 1 mM or 0.1 mM) was added, and the reaction was started by the addition of 0.5 ml enzyme solution. After incubation at 25 °C for 10 min, the reaction mixture was acidified with HCl to pH 3, and then 0.05 ml of 2 mM *n*-octanal in ethanol was added as an internal standard. To the mixture, 4 ml of 0.1% 2,4-dinitrophenylhydrazine containing acetic acid (0.5 M) was added. The resultant hydrazone derivatives were extracted with *n*-hexane and concentrated *in vacuo*. The concentrate was dissolved in chloroform and subjected to HPLC analysis. For quantification, calibration curves with authentic *n*-hexanal and (2*E*)-nonenal were constructed by using *n*-octanal as an internal standard. Analyses were performed with a HPLC (Shimadzu LC-6A) equipped with Zorbax-ODS column (Dupont;

4.6 mm ID × 25 cm) with detection at 350 nm. As a carrier solvent acetonitrile/tetrahydrofuran/water (80/1/19, v/v) at a flow rate of 1 ml/min was used [11].

Results and Discussion

Localization of LOX and HPO lyase activity

Most of the LOX activity (71%) was detected in the skin with outer flesh (preparation-A) which represented only 22% of the total weight of the tomato fruit (Table I). When the fleshy tissue was carefully removed from the skin to reduce the weight to 5.2% of the total (preparation-B), the activity was drastically reduced. These results suggests that LOX activity predominantly exists in the fleshy tissue adjacent to the skin.

HPO lyase activity was distributed evenly in skin with outer flesh, flesh without skin and locular material of the tomato fruit (Table II). HPO lyase in each part formed 0.7 to 1.5 μmol hexanal/g fr. wt./10 min, when 1 mM 13-HPO was used as a substrate.

Since the highest activities of LOX and HPO lyase existed in the skin with outer flesh (preparation-A), its homogenate was fractionated by sequential centrifugation. Most (73%) of LOX ac-

Table I. Tissue localization of lipoxygenase in tomato fruits.

Tissues	Ratio of weight [%]	Activity (U/g fr. wt.)	Yield of activity [%]
Whole tomato fruit	100	0.406	100
Skin with outer flesh	22.4[2]	1.286	71.0
	5.2[3]	1.573	20.2
Flesh without skin	57.9	0.203	29.0
Locular material	19.7	ND[1]	0

[1] Not detectable.
[2] Preparation-A.
[3] Preparation-B. (See Materials and Methods.)

Table II. Histological localization and substrate specificity of HPO lyase and aldehyde forming activities.

Tissues		Aldehyde forming activity (μmol/g fr. wt./10 min) Hexanal	(2*E*)-nonenal
Condition 1[1]			
Skin with outer flesh	LA	0.229	0.255
	13-HPO[3]	0.827	0
	9-HPO	0.350	0.007
Flesh without skin	LA	0.068	0.009
	13-HPO	0.700	0
	9-HPO	0.613	0
Locular material	LA	0.005	0
	13-HPO	1.501	0
	9-HPO	0.629	0
Condition 2[2]			
Skin with outer flesh	LA	0.046	0.012
	13-HPO	0.124	0
	9-HPO	0.044	0
Flesh without skin	LA	0.028	0.014
	13-HPO	0.113	0
	9-HPO	0.028	0
Locular material	LA	0	0.001
	13-HPO	0.097	0
	9-HPO	0.028	0

[1] Substrate was used at 1 mM.
[2] Substrate was used at 0.1 mM.
[3] See Materials and Methods.

tivity were recovered in the 20,000 × *g* pellet while 74% of HPO lyase activity were recovered in the 80,000 × *g* supernatant. To the best of our knowledge, this is the first report on a soluble HPO lyase. These results indicate that both LOX and HPO lyase activities exists in different subcellular fractions.

When mature green tomato fruits were stored at 25 °C, the color changed to red within 3 days. LOX activity was almost constant throughout the period, although it was reported that LOX activity of tomato fruits attached to the plant increased during ripening [12–14]. Positional specificity of dioxygenation of tomato LOX to LA was reported to be 5/95 (13-/9-HPO) [4, 14]. Similar results were obtained with LOX of tomato fruits in each period, and substantial changes of the ratio were not observed.

Substrate specificity of HPO lyase

When a low concentration of HPO (0.1 mM) was used, 13-HPO was a suitable substrate but 9-HPO was not a substrate for tomato fruit HPO lyase (Table II). When higher concentration of 9-HPO (1.0 mM) was added to the homogenate as a substrate, 0.1% of the substrate was cleaved to (2*E*)-nonenal. It must be pointed out that (2*E*)-nonenal was formed from LA whichever concentration was used. From this result, it is assumed that the substrate specificity of HPO lyase partly depends on a concentration of the respective substrate.

HPOs and aldehydes formed from exogenous substrates

The time course of HPOs and aldehydes formation was examined with LA as a substrate (Fig. 1). The amount of 9-*E,Z*-HPO, (2*E*)-nonenal and hexanal increased linearly over 20 min, 13-*Z,E*-HPO formation was terminated after 5 min. This result indicates that hexanal is produced from 13-*Z,E*-HPO which is a minor isomer of LOX products in tomato fruits and that the major isomer, 9-*E,Z*-HPO, was not substantially converted further but accumulated under the reaction condition employed here. Conversion of 9-*E,Z*-HPO by HPO lyase in the extract from skin with outer flesh to (2*E*)-nonenal was calculated to be 7.1% after 10 min and that of 13-*Z,E*-HPO to hexanal was 85% at the same time. The former value was much higher than the value of 0.1% when 1 mM 9-HPO was used (see Table II). From this calculation it is presumed that the manner of supply of 9-*E,Z*-HPO to HPO lyase performed by endogenous LOX is different from that when 9-HPO was added directly to the enzyme solution.

HPOs and aldehydes formed from endogenous substrates

Hexanal was always detected in crude homogenates of skin with outer flesh of tomato fruits even if no substrate was added. This indicates that C_6-aldehyde is formed from endogenous substrate existing in the intact tomato fruit. In order to exclude the formation of aldehydes from endogenous substrates during homogenization, whole tomato fruits were homogenized with 1 N HCl to denature lipid degrading enzymes. In the acid-denatured mixture 0.36 nmol/g fr.wt. ± 0.069 (mean ± SD, $n = 5$) of hexanal was still detected. We assume that this amount of hexanal has been formed and accumulated in intact tomato fruit.

When a tomato fruit was cut into eight fragments vertically at 5 °C and the fragments were incubated at 25 °C, the quantity of hexanal in the fragments increased to 1.914 nmol/g fr.wt. by

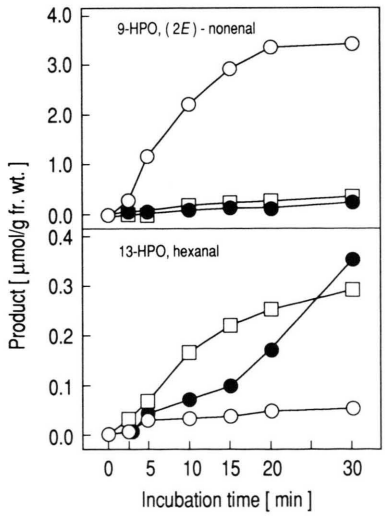

Fig. 1. HPOs and C_6- and C_9-aldehyde forming activity from linoleic acid as an exogenous substrate. Upper panel: Amount of 9-*E,Z*-HPO (○), 9-*E,E*-HPO (●) and (2*E*)-nonenal (□) formed from LA (1 mM). Lower panel: Amount of 13-*Z,E*-HPO (○), 13-*E,E*-HPO (●) and hexanal (□) formed from LA (1 mM).

20 min, then decreased to 1.642 nmol/g fr.wt. by 30 min (Fig. 2). This reduction may be caused by the volatile nature of the C_6-aldehyde. When a tomato fruit was homogenized with 50 mM potassium phosphate buffer (pH 6.3), which was the optimum pH of aldehyde forming activity, and incubated at 25 °C, the amount of hexanal in the homogenate rapidly increased to 18.63 nmol/g fr.wt. within 5 min and thereafter slightly increased to 21.10 nmol/g fr.wt. by 30 min. It has been previously reported that UV exposure can be employed to cause effects similar to those of pathogen infection on plant tissues and that phytoalexin elicited by a pathogen is also produced by UV irradiation [15, 16]. When a tomato fruit was cut vertically into two parts and the cut surface was exposed to UV irradiation for 10 min at 25 °C, the quantity of hexanal increased to 2.08 nmol/g fr. wt. This quantity was higher than that detected in the fragment cut into eight fragments and incubated without irradiation. When the surface of an intact fruit was exposed to UV irradiation for 10 min, the quantity of hexanal also increased to a level similar to that of fragment cut into eight pieces (Fig. 2). These results indicates that the amount of aldehydes formed endogenously in the fruit depends directly on the extent of injury. C_9-aldehyde was not detectable in the homogenate even after 30 min incubation of the fragment cut into eight pieces at 25 °C.

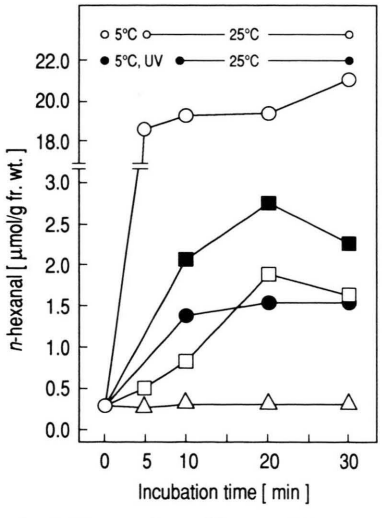

Fig. 2. Formation of hexanal from endogenous substrate in tomato fruit subjected to various forms of stress (cutting, homogenization or UV). Amount of hexanal in homogenate with 1 N HCl (△), fragments which were cut into 8 parts vertically (□), homogenate in 50 mM potassium phosphate buffer (pH 6.3) (○) from endogenous substrate. It took 5 min to cut and homogenize at 5 °C. Amount of hexanal in tomato fragments cut into 2 parts vertically and exposed UV irradiation at 25 °C (■) and that in UV irradiated intact tomato fruit (●). UV irradiation was carried out as described in Materials and Methods.

No HPO was detectable in tomato fruits homogenized in 1 N HCl or 50 mM potassium phosphate buffer (pH 6.3). When homogenate of whole tomato with 50 mM potassium phosphate buffer (pH 6.3) was incubated for 30 min at 25 °C, 18.63 nmol/g fr.wt. of 9-*E,Z*-HPO was detected but other HPO isomers were not detected. This content was too small when compared with the value calculated from the amount of hexanal formed during the same period based on the product specificity of LOX and the substrate specificity of HPO lyase shown above.

Whether C_6-aldehydes in intact tomato fruits are always released or it accumulates in the tissue is still unknown. But, results shown here indicate that stress, such as wound and UV irradiation, causes the expression of aldehyde forming activity in tomato fruits. That is, in intact tomato tissues LOX and HPO lyase either exist in latent forms or they are separated from substrates, but upon injury of the tissue, these enzymes catalyze aldehyde formation. Andersen and coworkers reported that LOX-HPO lyase mediated products in headspace of tobacco leaves increased as a result of injury [17]. It is thought that LOX and HPO lyase activities exist mostly latent in intact plant tissues and that they are activited upon some changes such as injury. The physiological roles of LOX and HPO lyase may be related to the response of tomato fruits to stress affecting the tissue. This assumption agrees with the observation that LOX activity was concentrated near the surface of the tomato fruit.

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