Non-Enzymatic Isomerization of 12-Hydroxy-(3*Z*)-dodecenal to the (2*E*)-Isomer after Enzymatic Cleavage of 13-Hydroperoxylinoleyl Alcohol in Tea Chloroplasts

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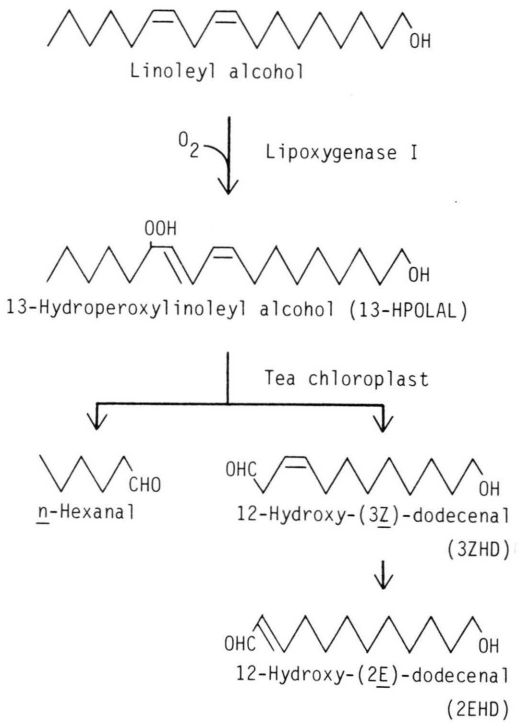
13-Hydroperoxylinoleyl Alcohol, 12-Hydroxy-(2*E*)-dodecenal, 12-Hydroxy-(3*Z*)-dodecenal, Tea Chloroplasts, Isomerization Factor

Incubation of synthetic 12-hydroxy-(3*Z*)-dodecenal (3ZHD) with tea chloroplasts revealed that 12-hydroxy-(2*E*)-dodecenal (2EHD) was the product of isomerization reaction of 3ZHD during the incubation. The isomerization was caused also by incubation of 3ZHD with the heat-inactivated chloroplasts. The isomerization activity was extracted with hot ethanol. These results indicate that the isomerization was caused by heat-stable factor(s) but not by enzyme(s) in tea chloroplasts.

Introduction

In tea chloroplasts, the cleavage products of 13-hydroperoxylinoleic acid (13-HPO) are detected as *n*-hexanal and C_{12}-oxo-acids, *i.e.*, 12-oxo-(9*Z*)-dodecenoic acid (9ZDA) and 12-oxo-(10*E*)-dodecenoic acid (10EDA) [1]. The cleavage is catalyzed by hydroperoxide lyase (HPO lyase). A mechanism of the enzymatic cleavage reaction was proposed by Gardner *et al.* [2]. We confirmed the mechanism by an experiment using ^{18}O-13-HPO and tea chloroplasts [3]. However, it was still unknown whether or not the isomerization of 9ZDA to 10EDA is caused enzymatically during incubation of 13-HPO with the chloroplasts.

HPO lyase in tea chloroplasts catalyzes cleavage of 13-hydroperoxylinoleyl alcohol (13-HPOLAL) as well as 13-HPO into *n*-hexanal and C_{12}-oxo-alcohols, *i.e.*, 12-hydroxy-(3*Z*)-dodecenal (3ZHD) and 12-hydroxy-(2*E*)-dodecenal (2EHD) as shown in Scheme 1. These unnatural oxo-alcohols can not be metabolized further and the products derived from the 13-HPOLAL are distinguished from endogenous products. Thus, we explored the isomerization of 3ZHD to 2EHD using 13-HPOLAL as a substrate for tea chloroplasts.



Scheme 1. Biosynthetic pathway of *n*-hexanal in tea leaves.

Results and Discussion

Detection of cleavage products of 13-HPOLAL

The cleavage products of 13-HPOLAL, 3ZHD and 2EHD, during incubation with tea chloroplasts were for the first time identified by high performance liquid chromatography (HPLC) analysis of the corre-

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sponding 2,4-dinitrophenylhydrazone derivatives (DNPHs) using authentic specimens as shown in Fig. 1.

The authentic samples synthesized through unequivocal routes, 3ZHD-, 3EHD-, and 2EHD-DNPHs were well resolved to three peaks at 16.35 min (peak a), 17.47 min (peak b), and 22.14 min (peak c), on Zorbax ODS, respectively. Two of peaks formed during incubation of 13-HPOLAL with tea chloroplasts, were coincident with 3ZHD (peak a) and 2EHD (peak c), respectively. However, the (3*E*)-isomer (peak b) was not detected.

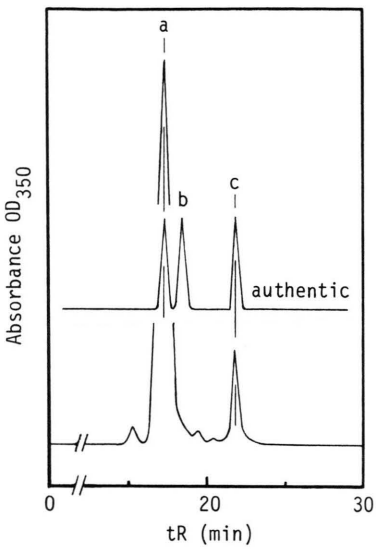

Fig. 1. Detection of 3ZHD and 2EHD produced during incubation of 13-HPOLAL by HPLC in form of 2,4-DNPHs. a: 3ZHD, b: 3EHD, c: 2EHD.

Isomerization activities of 3ZHD to 2EHD in tea chloroplasts

During incubation of 3ZHD at 35 °C in 50 mM Na-phosphate buffer (pH 7.0) without tea chloroplasts, the ratio of 2EHD to 3ZHD was 0.015 at 0 h, and only 0.025 after incubation for 1 h as shown in Fig. 2. However, rapid isomerization of 3ZHD to 2EHD were observed in both the non-treated and heat-treated (80 °C, 20 min) chloroplast fraction(s).

Isomerization factor(s) in tea chloroplasts

The isomerization factor(s) was extractable with hot ethanol from tea chloroplasts. While the ratio of

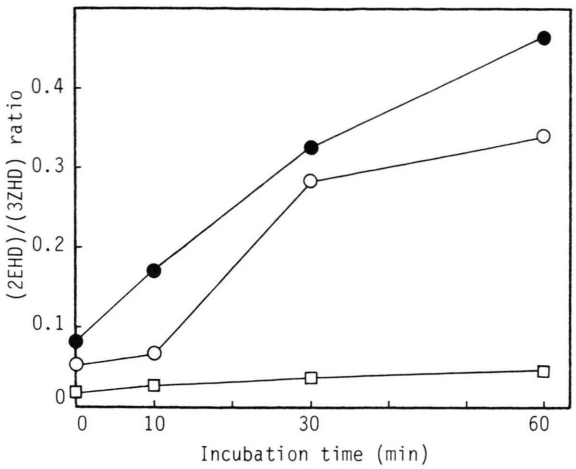

Fig. 2. Time course of isomerization of 3ZHD. (□): without chloroplast; (●): chloroplast; (○): heat-treated (80 °C, 20 min) chloroplast.

2EHD to 3ZHD were 0.031 after incubation of 3ZHD at 35 °C for 10 min with the Na-phosphate buffer alone, 0.083 with tea chloroplast suspension, 0.060 with the extract, and 0.034 with the extract residue were observed respectively. This result indi-

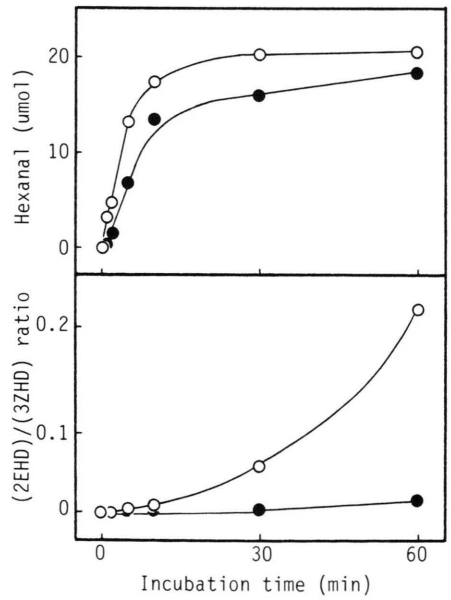

Fig. 3. Time course of hexanal formation and isomerization of 3ZHD from 13-HPOLAL in chloroplasts incubation. 13-HPOLAL and chloroplasts were incubated in 50 mM Na-phosphate buffer pH 7.0 at 35 °C (○) or in 25 mM Na-phosphate buffer pH 6.0 at 25 °C (●).

cates that the isomerization factor(s) in tea chloroplasts was not an enzyme but an ethanol soluble and stable compound(s) against treatment with hot ethanol. However, the isomerization of (3*Z*)-nonenal to (2*E*)-nonenal was reported to occur by an isomerase in cucumber fruits and seedling [4–7].

Effect of pH on isomerization

Effect of pH on the isomerization of 3ZHD to 2EHD in tea chloroplasts was observed (Fig. 4). As shown in Fig. 4, the isomerization with chloroplasts was accelerated as pH raised. The same pH-dependency of the isomerization was observed in the ethanol active extract described above (result not shown). From this result, it is suggested that the isomerization factor possess Lewis base group which is activated in alkaline media.

Time course of enzymatic cleavage reaction of 13-HPOLAL

The cleavage reaction of 13-HPOLAL catalyzed by HPO lyase in tea chloroplasts, was followed by the amount of formed hexanal using gas liquid chromatography (GLC, 5% PEG-20M). At the same time the isomerization of 3ZHD to 2EHD was followed by GLC analysis (5% Silicon OV-25) of the trimethylsilyl ether derivatives, 3ZHD-TMS and 2EHD-TMS. As shown in Fig. 3, the formation of hexanal reached a plateau after 15 min of incubation at 35 °C, whereas the isomerization of 3ZHD to 2EHD was gradually increased as incubation proceeds up to 60 min when 13-HPOLAL and tea chloroplasts were incubated in 50 mM Na-phosphate buffer (pH 7.0) at 35 °C. When they were incubated in 25 mM Na-phosphate buffer (pH 6.0) at 25 °C, *n*-hexanal and 3ZHD were formed, whereas the isomerization ratio (2EHD/3ZHD) extensively decreased as shown in Fig. 3. This indicates that the cleavage reaction of 13-HPOLAL by HPO lyase forms 3ZHD but not 2EHD as a primary product and 2EHD was formed by the isomerization of 3ZHD after the cleavage reaction during longer incubation with tea chloroplasts. From this finding, it is clarified that HPO lyase maintains the (*Z*)-configuration of double bond between C-9 and C-10 of substrate. These results support that tea HPO lyase recognize and retain the (*Z*)-configuration at C-9 position of 13-HPOLAL and catalyze the cleavage reaction without any geometrical isomerization at this position. After the 3ZHD was liberated from the enzyme, the geometrical isomerization of 3ZHD to 2EHD was caused by the ethanol soluble isomerization factor(s) having functional group(s) which is activated in alkaline media.

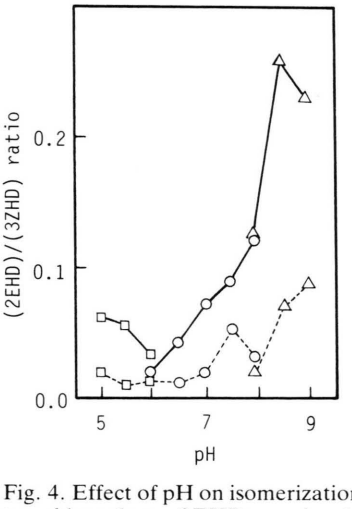

Fig. 4. Effect of pH on isomerization of 3ZHD to 2EHD in tea chloroplasts. 2EHD was incubated with chloroplasts (–) or without chloroplasts (·····) in buffer at 35 °C for 10 min. (□): McIlvain's buffer, (○): phosphate buffer, (△): pyrophosphate buffer.

Experimental

Preparation of chloroplasts

Fresh leaves of tea (*Camellia sinensis* cv. Yabukita) were homogenized for 3 min in a chilled blender with 3 volumes of McIlvaine's buffer (pH 6.3) containing 0.4 M sucrose [8]. The homogenate was filtered through 4 layers of cheese-cloth and chloroplast fraction was collected by centrifugation at 4000 × *g* for 10 min and was stored at −20 °C in 2.1 M sucrose. Before use, the stored chloroplast fraction was washed twice with 50 mM Na-phosphate buffer (pH 7.0).

Preparation of 13-hydroperoxylinoleyl alcohol

13-(9*Z*,11*E*)-HPOLAL (containing 6.0% of geometrical and positional isomers of the hydroperoxide) was prepared from linoleyl alcohol by soybean lipoxygenase (Sigma, Type I). 13-(9*E*,11*E*)-HPOLAL (containing 5.8% of geometrical and positional isomers of the hydroperoxide) was obtained by separation of crude HPOLAL (preparing by the lipoxygenase as above) using HPLC (equipping Zorbax SIL ∅ 4.6 mm × 250 mm).

Preparation of authentic specimens

3ZHD was synthesized by the coupling 1,8-octanedioic acid to acetylene, and bromoacetaldehyde diethylacetal, followed by (*Z*)-stereoselective hydrogenation with Lindlar catalyst, and subsequent selective removal of the protecting groups. 3EHD was synthesized from a synthon, 12-(2-tetrahydropyranyloxy)-9-dodecyne diethylacetal, by (*E*)-selective hydrogenation with Na in liquid NH_3. 2EHD was obtained from ethyl 10-undecenoate and ethyl bromoacetate, 12-hydroxy-(2*E*)-dodecenal was obtained by ozonolysis, followed by (*E*)-selective Wittig reaction, reduction with AlH_3, and selective oxidation with MnO_2 (will be reported elsewhere).

Extraction of isomerization activity from tea chloroplast

After a suspension of chloroplast (7.2 g) in 100 ml of ethanol was refluxed for 24 h, the resulting residue was separated with a glass filter. The filtrated solution was evaporated *in vacuo* to give an active extract (0.6 g).

Analyses and identification of cleavage products of 13-HPOLAL

13-Hydroperoxylinoleyl alcohol (24 μmol) was added into 48 ml of chloroplast suspension (0.01 g/ml, 50 mM Na-phosphate buffer pH 7.0). After the suspension was incubated at 35 °C for 10 min with stirring, a saturated $(NH_4)_2SO_4$ solution (12 ml) was added to the reaction mixture to stop the reaction. The suspension was centrifuged at 15,000 × *g* for 20 min. To the supernatant 0.1% 2,4-dinitrophenylhydrazine solution in ethanol (pH adjusted to 4.0 with acetic acid) was added and stirred for 12 h. The ether extract was evaporated *in vacuo* to leave a residue (0.25 ml), which was filled up to 0.5 ml with ether. The extract was purified by silica gel thin layer chromatography (pet. ether/ether/AcOH 80/70/0.5). The region of R_f 0.12–0.18 was scratched and extracted with ether. Evaporation of the ether extract *in vacuo* gave a mixture of products. The mixture was filled up to 0.2 ml with $CHCl_3$ and 3ZHD, 3EHD, and 2EHD were quantitatively analyzed by HPLC (Hitachi 655A; Zorbax ODS ∅ 4.6 mm × 15 cm; eluent acetonitrile/water/THF (62/37/1); flow rate 1.0 ml/min; detector UV (OD_{350})) using authentic specimens.

Isomerization activities of 3ZHD to 2EHD in tea chloroplasts

A suspensions of chloroplasts or heat-treated chloroplasts (each 0.01 g/ml) in 4.0 ml of 50 mM Na-phosphate buffer (pH 7.0) were incubated with 3ZHD (2.0 mmol) at 35 °C for given periods. Aldehydes formed were analyzed by HPLC as described above.

Time course of enzymatic cleavage reaction of 13-HPOLAL

Chloroplast suspensions (48 ml, 50 or 25 mM, pH 7.0 or 6.0) and 13-HPOLAL (each 24 μmol) were incubated at 35 or 20 °C. After incubations for given periods, the headspace vapors (each 6.0 ml) in reaction flasks (each 300 ml) were analyzed by GLC (column: 5% PEG 20 M on celite 545 ∅ 3.0 mm × 3.0 m column temp. 100 °C, inj. and det. temp. 150 °C; flow rate 60 ml/min) and formed hexanal was estimated. On the other hand, $NaBH_4$ (1.0 mmol) was added into the reaction solution immediately after the incubation, and was stirred for 30 min. After addition of $(NH_4)_2SO_4$ (10 g) into the suspension, the suspension was centrifuged at 15,000 × *g* for 20 min. The resulting supernatant was extracted with ether. The extracts was evaporated *in vacuo* to leave a concentrate, which was filled up to 0.5 ml with ether. Products in the extracts were analyzed by GLC (column: 5% Silicon OV-25 on Chromosorb W AW ∅ 3.0 mm × 1.0 m, column temp. 100–200 °C (4 °C/min), inj. and det. temp. 230 °C; flow rate 60 ml/min) after addition of TMS-HF (150 μl).

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