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Autoxidation Products of Phytofluene in Liposome and Conversion of Phytapentaenal to 4,5-Didehydrogeranyl Geranoic Acid in Pig Liver Homogenate

– Research Note –

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Abstract

The cleavage products formed by autoxidation of phytofluene were evaluated in order to elucidate possible oxidation products of phytofluene under oxidative conditions. Phytofluene solubilized at 50 μM in liposomal suspension was oxidized by incubating at 37°C for 72 h. Among a number of oxidation products formed, five products in the carbonyl compound fraction were identified as 6,10,14-trimethylpentadeca-3,5,9,13-tetraen-2-one, phytapentaenal, 5,9,13,17-tetramethyloctadeca-2,4,6,8,12,16-hexaenal, 5,9,13,17-tetramethyloctadeca-2,4,8,12,16-pentaenal, 2,7,11,15,19-pentamethylicosa-2,4,6,10,14,18-hexaenal and 4,9,13,17,21-pentamethyldocosa-2,4,6,8,12,16,20-heptaenal. These correspond to a series of products formed by cleavage in the respective eight conjugated double bonds of phytofluene. Also, 4,5-didehydrogeranyl geranoic acid was formed by autoxidation of phytofluene in liposomal suspension. The pig liver homogenate had the ability to convert phytapentaenal to 4,5-didehydrogeranyl geranoic acid, comparable to the conversion of all-*trans*-retinal to all-*trans*-retinoic acid. These results suggest that phytofluene is cleaved to a series of long-chain and short-chain carbonyl compounds under the oxidative condition *in vitro* and that phytapentaenal is further enzymatically converted to 4,5-didehydrogeranyl geranoic acid.

Key words: *phytofluene, autoxidation, cleavage products*

INTRODUCTION

Provitamin A carotenoids are metabolized to vitamin A through enzymatic cleavage at central double bonds to retinal in intestinal cells of vertebrates (1,2). Eccentric cleavage, by which double bonds of provitamin A were cleaved at random positions, has also been proposed as an additional pathway for retinoid synthesis (3). The cleavage products such as retinal and β-apo-carotenals with different carbon chain length have been known to be produced from β-carotene by non-enzymatic oxidation under various conditions: autoxidation in solvents, oxidation with peroxy radical initiators, singlet oxygen and cigarette smoke, and cooxidation by lipoxygenase (1-7). Canthaxanthin was also reported to give a series of cleavage products by oxidation with nickel peroxide (8). Moreover, 4-oxo-retinoic acid was identified as an oxidation product of canthaxanthin incubated in a cell culture medium and was found to activate RARβ gene promoter and to enhance gap junctional communication (9,10). The oxidation product of β-carotene, 5,8-endoperoxy-2,3-dihydro-β-apocarotene-13-one, has shown to inhibit growth and cholesterol synthesis of breast cancer cells (11). A urinary metabolite of canthaxanthin in rats was identified as 3-hydro-4-oxo-7,8-dihydro-β-ionone (12), and one of astaxanthine metabolites in primary culture of rat liver was found to be a glucuronide of 3-hydroxy-4-oxo-β-ionone (13). These reports suggest that oxidation of carotenoids including non-provitamin A carotenoids gives eccentric cleavage products in biological tissues and that some of cleavage products are biologically active compounds as retinoids.

Phytofluene, one of the non-provitamin A carotenoids, was isolated from carrot oil (14) and tomato related products such as tomato oleoresin, juice and paste (15). In this study, the formation of cleavage products from phytofluene under oxidative conditions was evaluated *in vitro*, in order to investigate possible oxidation products formed in biological systems.

METERIALS AND METHODS

Materials

Phytofluene and 4,5-didehydrogeranyl geranoic acid was obtained from National Food Research Institute, Japan. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine was purchased from Sigma Chemical Co. (St. Louis, MO, USA.). HPLC-grade acetonitrile was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). *d*-α-Tocopherol was purchased from Wako Co. (Tokyo, Japan). Other chemicals and solvents used were of analytical reagent grade.

Autoxidation of phytofluene

Autoxidation of phytofluene, solubilized at 50 μM in liposomal suspension, was carried out by incubating under atmospheric oxygen at 37°C for 72 h. Phytofluene solubilized in a liposomal suspension was prepared as follows. Five μmol of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 50 nmol of phytofluene dissolved in dichloromethane were mixed in a test tube (1.3×10 cm), and the solvent was removed under a stream of argon and then *in vacuo* for 30 min. The residue

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was dispersed in 1 mL of 0.01 M Tris-buffer, pH 7.4, containing 0.5 mM of diethylenetriaminepentaacetic acid by mixing with a vortex mixer for 1 min and sonicating with a Branson 1210J ultrasonifier (Branson, Danbury, CT, USA) for 30 sec at an ambient temperature. Phytofluene solubilized in liposome was incubated at 37°C with continuous shaking at 120 rpm. After incubation, 1 mL of 0.02% *d*-α-tocopherol/ethanol was added and the mixture was stored at -80°C until extraction.

Extraction of oxidation products

Residual phytofluene and its oxidation products formed in liposomal suspension were extracted as follows. Phytofluene was extracted three times with 2 mL of hexane from the stored mixture. The combined extract was evaporated to dryness under argon gas, and the residue was dissolved in 2 mL of acetone. A 20 µL aliquot of the final extract was subjected to an HPLC analysis of phytofluene. Carbonyl compounds were extracted as in case of phytofluene, and extract was dissolved in 300 µL of hexane/ethylacetate (99:1, v/v). The extract was fractionated with a Bond Elut solid phase cartridge, and subjected to HPLC analysis as described below. For 4,5-didehydrogeranyl geranoic acid, 2 mL of 1 N NaOH was added to the stored mixture (40 mL). The mixtures were washed three times with 40 mL of hexane. The aqueous phase was acidified with 600 µL of 6 N HCl and 4,5-didehydrogeranyl geranoic acid was extracted three times with 40 mL of hexane. The combined extracts were evaporated to dryness under argon gas and dissolved in 200 µL of hexane/ethylacetate (92:8, v/v), and subjected to HPLC analysis as described below.

Conversion of phytapentaenal to 4,5-didehydrogeranyl geranoic acid by liver homogenate

The pig liver was homogenized with a Potter Elvehjem homogenizer in five volumes of 0.1 M KH_2PO_4-K_2HPO_4 buffer, pH 7.4 and 0.15 M KCl. A supernatant solution of homogenate, after centrifugation at 9,000 g for 30min, was used to evaluate metabolic activity of liver homogenate for conversion of phytapentaenal to 4,5-didehydrogeranyl geranoic acid under the modified conditions reported previously (16). The reaction mixture contained 10 M phytapentaenal or all-*trans*-retinal, 0.5 M Tricine-KOH buffer, pH 8.0, 150 mM KCl, 2 mM NAD, 2 mM DTT, and homogenate (9.1 mg protein) in a total volume of 1 mL. After preincubation at 37°C for 10 min, the reaction was initiated by adding 5 µL of 2 mM phytapentaenal or all-*trans*-retinal in dimethyl sulfoxide. The mixture was then incubated at 37°C for 60 min. The reaction was stopped by adding 3 mL of 0.025 N KOH in ethanol. The mixture was washed two times with 6 mL hexane. The aqueous phase was then acidified with 0.12 mL of 6 N HCl and acycloretinoic acid was extracted three times with 6 mL of hexane containing 0.001% butylhydroxytoluene. The combined extract was dried under a stream of argon gas, dissolved in 200 µL of acetonitrile/methanol/water (70:20:10, v/v/v) containing 0.1% acetic acid and subjected to HPLC analysis. Protein concentration in the homogenate was determined by the Bradford method (17) with the bovine serum albumin as standard.

HPLC analyses

Oxidative cleavage of phytofluene was analyzed by HPLC on a TSK-GEL ODS 80 TS (Tosoh, Co. Tokyo) 4.6×250 mm, attached to precolumn (2×20 mm) of Pelliguard LC-18 (Supelco, Inc., Bellefonte, PA). The solvent system consisted of acetonitrile/methanol/water (75:15:10, v/v/v) containing 0.1% ammonium acetate (solvent A) and methanol/ethylacetate (70:30, v/v) containing 0.1% ammonium acatate (solvent B). A linear gradient from solvent A (100%) to solvent B (100%) was applied for 10 min at a flow rate of 1 mL/min, followed by isocratic elution with solvent B (100%) for an additional 10 min. They were monitored at each λ max with the photodiode array detector.

4,5-Didehydrogeranyl geranyl acid was analyzed by HPLC on the same column as above with acetonitrile/methanol/water (70:20:10, v/v/v) containing 0.1% acetic acid was used as a mobile at flow rate of 1 mL/min. It was monitored at 315 nm with photodiode array detector.

LC-MS analyses

To identify the cleavage products obtained from ozonolysis of phytofluene, the carbonyl compound fraction was subjected to a LC-MS analysis. Positive ion mass spectra were obtained using a Model M-1200AP mass spectrometer (Hitachi Co., Tokyo, Japan) equipped with an APCI-MS interface, and a Model L-7100 gradient HPLC system (Hitachi Co.).

RESULTS

A number of peaks with absorption in UV-VIS region appeared in HPLC profiles of the crude extracts after autoxidation of phytofluene in liposomal suspension. After fractionation of the crude extract on a silica column (Bond Elut SI), the eluate with hexane/ethylacetate (95:5, v/v) showed clearly the presence of a series of carbonyl compounds which were formed by cleavage of conjugated double bonds of phytofluene.

Figure 1 shows an HPLC chromatogram of the carbonyl compound fraction obtained from the oxidized phytofluene in liposomal suspension. The peaks from 1 to 6 were assigned by comparing retention time and UV-VIS spectra with those of the reference cleavage products prepared by ozonolysis of phytofluene (18). They had a characteristic bell-shape spectrum of UV-VIS absorption similar to that of all-*trans*-retinal, but different λ max (peak 1, 290 nm; peak 2, 335 nm; peak 3, 365 nm; peak 4, 290 nm; peak 5, 335 nm; peak 6, 365 nm), based upon its UV-VIS spectra and $[M+H]^+$ ion.

Peak 2 (phytapentaenal) was the cleavage product at central double bond of phytofluene. Peak 1 (6,10,14-trimethylpentadeca-3,5,9,13-tetraen-2-one) and peak 3 (5,9,13,17-tetramethyloctadeca-2,4,6,8,12,16-hexaenal) were the cleavage products at C13-C14 double bond. Peak 4 (5,9,13,17-tetramethyloctadeca-2,4,8,12,16-pentaenal) was the cleavage products at C13′-

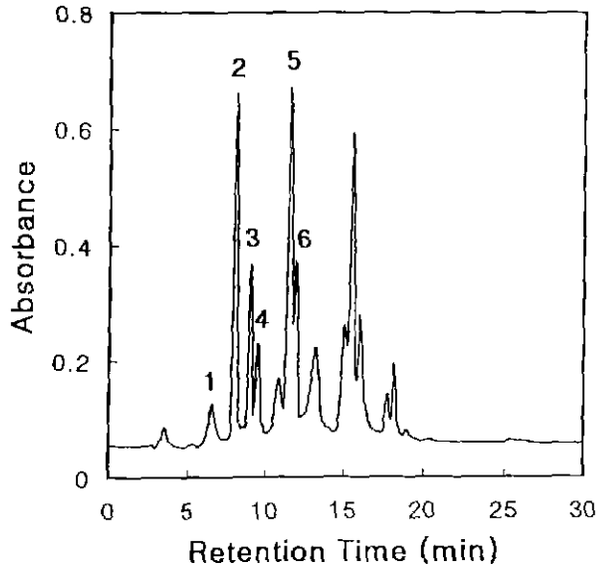

Fig. 1. HPLC chromatogram for autoxidation products of phytofluene Phytofluene was solubilized at 50 μM in the liposomal suspension of 5 mM dimyristoylphospatidylcholine and incubated at 37°C for 72 h. The cleavage products extracted from incubation mixture were separated by HPLC on a TSK-GEL ODS 80Ts, 4.6× 250 mm and were monitored at 335 nm with the photodiode array detector as described in Materials and Methods. Peak 1, 6,10,14-trimethylpentadeca-3,5,9,13-tetraen-2-one (6.7 min); peak 2, phytapentaenal (8.1 min); peak 3, 5,9,13,17-tetramethyloctadeca-2,4,6,8,12,16-hexaenal (9.1 min); peak 4, 5,9,13,17-tetramethyloctadeca-2,4,8,12,16-pentaenal(9.7 min); peak 5, 2,7,11,15,19-pentamethylicosa-2,4,6,10,14,18-hexaenal (11.6 min); peak 6, 4,9,13,17,21-pentamethyldocosa-2,4,6,8,12,16,20-heptaenal (12.0 min).

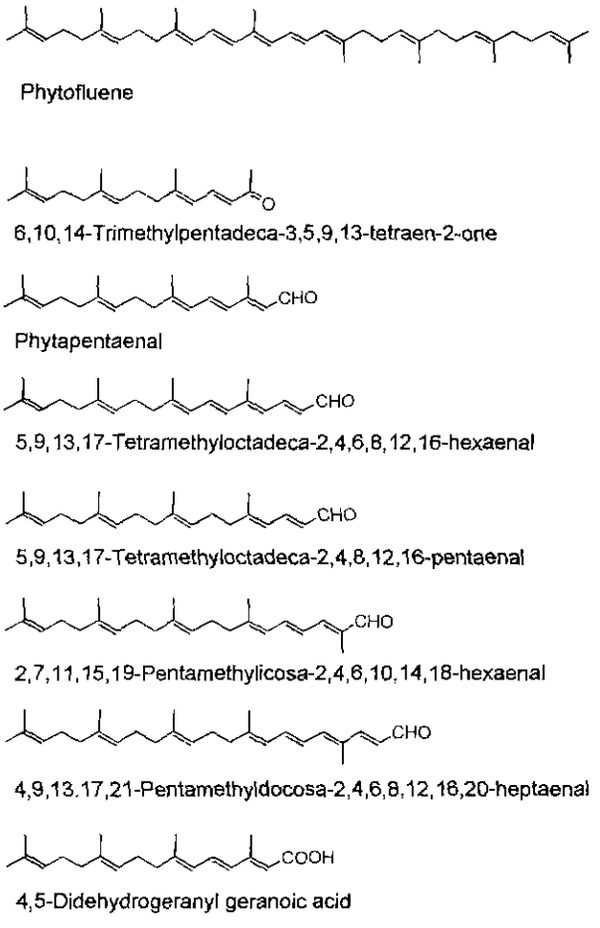

Fig. 2. Structure of autoxidation products of phytofluene.

C14′ double bond. Long chain compounds such as 2,7,11,15,19-pentamethylicosa-2,4,6,10,14,18-hexaenal (peak 5) and 4,9,13,17,21-pentamethyldocosa-2,4,6,8,12,16,20-heptaenal (peak 6) were also detected. Their structures are shown in Fig. 2.

The extract under acidic conditions from the oxidized phytofluene in liposomal suspension contained the oxidation products with the same retention time and UV-VIS spectra as standard 4,5-didehydrogeranyl geranoic acid in HPLC analysis (18).

To evaluate the metabolic conversion of phytapentaenal into 4,5-didehydrogeranyl geranoic acid in tissue homogenate, pig liver homogenate was incubated with 10 M phytapentaenal. Phytapentaenal and all-*trans*-retinal were efficiently converted to the corresponding carboxylic acids by incubating with pig liver homogenate as shown in Table 1. No conversion in the control incubations of minus homogenate and heat-inactivated homogenate indicated the enzymatic catalysis. The conversion ratio of the incubated phytapentaenal and retinal to the acids were 95.5% and 82.4%, respectively, after incubation for 60 min.

Table 1. Conversion of phytapentaenal to 4,5-didehydrogeranyl geranoic acid by pig liver homogenate[1)]

	4,5-Didehydrogeranyl geranoic acid (pmol/mg protein/h incubation)	Retinoic acid
Complete incubation	984.2±34.3	867.8±4.7
Zero-time control	12.5±4.6	32.6±2.5
Minus homogenate control	23.6±3.6	25.7±2.6
Minus substrate control	ND[2)]	27.6±4.3
Heat-inactivated homogenate control[3)]	2.6±0.0	15.7±4.2

[1)]Phytapentaenal and retinal were incubated at 10 μM with pig liver homogenate (9.1 mg protein/mL) at 37°C for 10 min and amounts of 4,5-didehydrogeranyl geranoic acid in the extracts were measured by HPLC. Values are expressed as means±standard deviations of triplicate incubations.
[2)]ND. less than 0.1.
[3)]Treatment of heat-inactivated homogenate was 100°C for 10 min.

DISCUSION

Carotenoids are highly susceptible to oxidation under certain oxidative conditions to produce a number of compounds. Recent studies have indicated that oxidation products of carotenoids showed biological effects *in vitro* (9,10). Some oxidation products might participate in their biological effects reported in animal models and cell culture systems (13). Thus we have evaluated the *in vitro* oxidation products of phytofluene, a typical non-provitamin A carotenoid, and have identified a homologous series of carbonyl compounds formed by cleavage at the conjugated double bonds and an analogue of retinoic acid.

A number of oxidation products have appeared by autoxidation of phytofluene under oxidative conditions. Phytofluene was solubilized as a micelle in a phospholipid membrane of liposome as a model of biological tissue. The carbonyl compounds were clearly separated in HPLC analysis after fractionation on a small silica column. The six products identified were 6,10,14-trimethylpentadeca-3,5,9,13-tetraen-2-one, phytapentaenal, 5,9,13,17-tetramethyloctadeca-2,4,6,8,12,16-hexaenal, 5,9,13,17-tetramethyloctadeca-2,4,8,12,16-pentaenal, 2,7,11,15,19-pentamethylicosa-2,4,6,10,14,18-hexaenal and 4,9,13,17,21-pentamethyldocosa-2,4,6,8,12,16,20-heptaenal.

These results were consistent with the autoxidation of β-carotene in organic solvent. Mordi et al. have proposed a cleavage reaction at the double bond through a dioxetane from a peroxy radical of β-carotene (1,2). The results in our study and in the previous reports suggest that the cleavage reaction to carbonyl compounds at the conjugated double bond by autoxidation, radical mediated oxidation, and singlet oxygen occur in any carotenoid with a long-chain of conjugated double bonds. Mordi et al. suggested the formation of retinoic acid by autoxidation of β-carotene (2). Nikawa et al. found 4-oxoretinoic acid as oxidation products of canthaxanthine when incubated in a cell culture medium (9). In the previous study, we had clearly demonstrated the formation of 4,5-didehydrogeranyl geranoic acid from autoxidation of phytofluene in 5% Tween 40 solution (18). We also found formation of 4,5-didehydrogeranyl geranoic acid from phytofluene under liposomal suspension as aqueous media (18). Therefore, 4,5-didehydrogeranyl geranoic acid formation would be if phytofluene is oxidized in biological tissues. We found that the liver homogenate had metabolic activity for conversion of phytapentaenal to 4,5-didehydrogeranyl geranoic acid, comparable to that for conversion of retinal to retinoic acid, although we did not evaluate whether other carbonyl compounds were converted to the corresponding acids. Thus, 4,5-didehydrogeranyl geranoic acid is potentially formed from phytapentaenal, when phytofluene is oxidized in biological tissues.

In this study, we have demonstrated *in vitro* formation of 4,5-didehydrogeranyl geranoic acid from autoxidation of phytofluene. Araki et al. found that 4,5-didehydrogeranyl geranoic acid and retinoic acid could strongly activate a RARE of RAR-β gene and RXRE of human cellular retinol-binding protein Type II gene, could bind to cellular retinoic acid binding protein (19).

The results suggest the *in vitro* formation of the cleavage products of carotenoids. The detection and identification of oxidation products of carotenoids formed *in vivo* are needed to clarify their possible involvement in biological effects of carotenoids.

ACKNOWLEDGEMENTS

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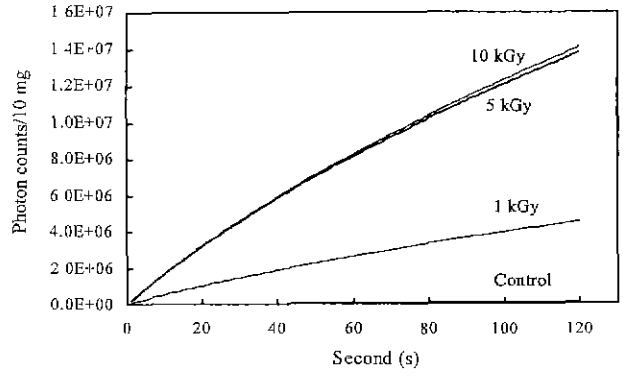
Erratum Notice

Corrections should be made for the paper entitled as "The Use of Pulsed Photostimulated Luminescence (PPSL) and Thermoluminescence (TL) for the Detection of Irradiated Perilla and Sesame Seeds" published in the *J. Food Sci. Nutr.* Vol. 5, No. 3, p. 142 (2000) such as:



A) Mineral seperated from irradiated sesame seeds

Fig. 1. PPSL curves of minerals seperated from irradiated sesame and perilla seeds at various doses.