Food Sci. Biotechnol. Vol. 14, No. 3, pp. 424 ~ 427 (2005)

RESEARCH NOTE

Food Science and Biotechnology

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Oxidative Cleavage Products Derived from Phytofluene by Pig Liver Homogenate

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Abstract The cleavage products formed by the autoxidation of phytofluene were evaluated in order to elucidate possible oxidation products of phytofluene in the oxidative condition. Among a number of oxidation products formed, the following five in the carbonyl compound fraction were identified: 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. In addition, 4,5-didehydrogeranyl geranoic acid was formed by the autoxidation of phytofluene in liposomal suspension. The pig liver homogenate was able to convert phytapentaenal to 4,5-didehydrogeranyl geranoic acid, in a manner comparable to the conversion of all-*trans*-retinal to all-*trans*-retinoic acid. These results suggest firstly that phytofluene is cleaved into a series of long-chain and short-chain carbonyl compounds under the oxidative condition *in vitro* and secondly that phytapentaenal is further enzymatically converted to 4,5-didehydrogeranyl geranoic acid.

Key words: phytofluene, autoxidation, cleavage products, pig liver homogenate, *in vitro*

Introduction

Provitamin A carotenoids are metabolized to vitamin A through enzymatic cleavage at the central double bond to retinal in intestinal cells of vertebrates (1, 2). Eccentric cleavage, by which the double bonds of provitamin A were cleaved at random position, has also been proposed as an additional pathway for retinoid synthesis (3). 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 including autoxidation in solvents, oxidation with peroxy radical initiators, singlet oxygen and cigarette smoke, and cooxidation by lipoxygenase (4-7). The oxidation product of β-carotene, 5,8-endoperoxy-2,3-dihydro-β-apocarotene-13-one, has been shown to inhibit growth and cholesterol synthesis of breast cancer cells (8). A urinary metabolite of canthaxanthin in rats was identified as 3-hydro-4-oxo-7,8-dihydro-β-ionone (9), and one of the astaxanthine metabolites in the primary culture of rat liver was found to be a glucuronide of 3-hydroxy-4-oxo-β-ionone (10). These reports suggest that the oxidation of carotenoids, including non-provitamin A carotenoids, produces eccentric cleavage products in biological tissues and that some cleavage products are biologically active compounds as retinoids.

Phytofluene, a non-provitamin A carotenoid, was isolated from carrot oil (11) and tomato-related products such as tomato oleoresin, juice and paste (12). In this study, the formation of cleavage products from phytofluene under oxidative condition was evaluated *in vitro*, in order to investigate possible oxidation products formed in biological systems.

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Received November 19, 2004; accepted May 17, 2005

Materials and Methods

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. Briefly, 5 μmol of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 50 nmol of phytofluene dissolved in dichloro-methane were mixed in a test tube (1.3 × 10 cm), and the solvent was removed under a stream of argon and then held *in vacuo* for 30 min. The residue 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, U.S.A.) for 30 sec at ambient. 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 the extractions were performed.

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 underwent HPLC analysis of phytofluene. Carbonyl compounds were extracted as in the case of phytofluene, and the 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 underwent HPLC analysis as described below. For 4,5-didehydrogeranyl geranoic acid, 2 mL of 1 N NaOH was added to 40 mL of the stored mixture. 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, dissolved in 200 μL of hexane/ethylacetate (92:8, v/v), and underwent HPLC analysis as described below.

Bioconversion of phytapentaenal 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 30 min, was used to evaluate the metabolic activity of the liver homogenate for conversion of phytapentaenal to 4,5-didehydrogeranyl geranyl acid under the modified conditions reported previously (13). 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 of 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 underwent HPLC analysis. Protein concentration in the homogenate was determined by the Bradford method (14) with 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, Japan), 4.6 × 250 mm, attached to a precolumn (2 × 20 mm) of Pelliguard LC-18 (Supelco, Inc., Bellefonte, PA, USA). 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 acetate (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 absorption spectra ëmax 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 and was used as a mobile at a flow rate of 1 mL/min. It was monitored at 315 nm with the photodiode array detector.

LC-MS analyses To identify the cleavage products obtained from ozonolysis of phytofluene, the carbonyl compound fraction underwent 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 and Discussion

A number of peaks with absorption in the UV-VIS region appeared in the HPLC profiles of the crude extracts after autoxidation of phytofluene in liposomal suspension. Figure 1 shows the 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 times and UV-VIS spectra with those of the reference cleavage products prepared by ozonolysis of phytofluene (15). They had a characteristic bell-shape spectrum of UV-VIS absorption similar to that of all-*trans*-retinal, but with different ëmax (290, 335, 365, 290, 335, and 365 nm for peaks 1-6, respectively), based upon its UV-VIS spectra and $[M+H]^+$ ion. Peak 2 (phytapentaenal) was the cleavage product at the central double bond of phytofluene. Peaks 1 (6,10,14-trimethylpentadeca-3,5,9,13-tetraen-2-one) and 3 (5,9,13,17-tetramethyloctadeca-2,4,6,8,12,16-hexaenal) were the cleavage products at the C13-C14 double bond. Peak 4 (5,9,13,17-tetramethyloctadeca-2,4,8,12,16-pentaenal) was the cleavage product at the C13'-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 Figure 2. The product extracted under acidic condition from the oxidized phytofluene in liposomal suspension contained oxidation products that had the same retention time and UV-VIS spectra as standard 4,5-didehydrogeranyl geranoic acid in

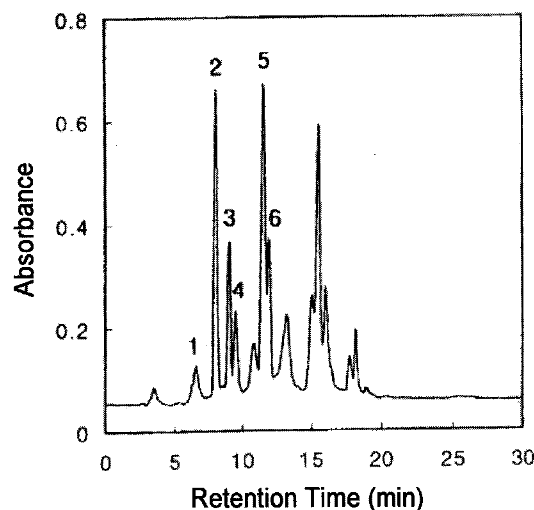

Fig. 1. HPLC chromatogram for autoxidation products of phytofluene. Phytofluene was solubilized at 50 μM in the liposomal suspension of 5 mM dimyristoylphosphatidylcholine and incubated at 37°C for 72 h. The cleavage products extracted from the incubation mixture were separated by HPLC on a TSK-GEL ODS 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).

HPLC analysis (15).

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. A lack of 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 was 95.5% and 82.4%, respectively, after incubation for 60 min. Carotenoids are highly susceptible to oxidation under certain oxidative conditions and produce a number of compounds. Recent studies have indicated that the oxidation products of carotenoids showed biological effects *in vitro* (16, 17). Some of the oxidation products might participate in the biological effects that have been reported in animal models and cell culture systems (18). Thus we evaluated the *in vitro* oxidation products of phytofluene, a typical non-provitamin A carotenoid, and 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 appeared by autoxidation of phytofluene under oxidative condition. Phytofluene was solubilized as a micelle in a phospholipid membrane of liposome as a model of biological tissues. In HPLC analysis, the carbonyl compounds were clearly separated after fractionation on a small silica column. The following six products were identified:
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.

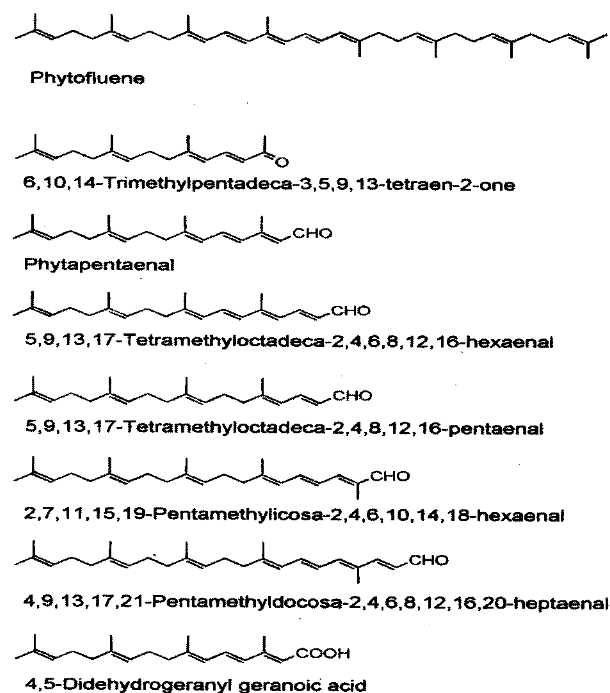

Fig. 2. Structure of autoxidation products from phytofluene.

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 peroxy radical of β-carotene (1,2).

The results in our study, in addition to those of previous reports, suggest that the cleavage reaction to carbonyl compounds at the conjugated double bond, which proceeds by autoxidation, radical mediated oxidation, and singlet oxygen, occurs 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-oxo-retinoic acid as the oxidation products of canthaxanthin when incubated in a cell culture medium (9). We found that the metabolic activity of the liver homogenate for the conversion of phytapentaenal to 4,5-didehydrogeranyl geranoic acid was comparable to that for the 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 geranyl acid is potentially formed from phytapentaenal, when phytofluene is oxidized in biological tissues.

The results suggest the *in vitro* formation of the cleavage products of carotenoids. Further study should involve the detection and identification of the oxidation products of carotenoids formed *in vivo* in order to clarify their possible involvement in the biological effects of carotenoids.

Acknowledgments

This work was supported by the Food Industrial Technology Research Center (RRC) at Mokpo National University, Korea, and the National Food Science Institute, Japan.

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