Note

Synthesis of a Novel Phosphate Ester of a Vitamin E Derivative and Its Antioxidative Activity

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A novel phosphate ester containing a chromanol structure was synthesized from 1,2-diacetyl-sn-glycero-3-phospho-2'-hydroxyethyl-2',5',7',8'-tetramethyl-6'-hydroxychroman (PCh) by hydrolysis catalyzed by phospholipase C from Bacillus cereus. The structure of the product was found by spectral analyses to be 2-(2',5',7',8'-tetramethyl-6'-hydroxychromanyl)ethylphosphate (Ch-P). Ch-P was highly soluble in the aqueous phase at neutral pH values and exerted higher antioxidative activity than α-tocopherol and PCh in the Fe(III)/ascorbic acid-catalyzed peroxidation of a fish oil emulsion and the autoxidation of a rat brain homogenate.

Key words: vitamin E; phospholipase C; antioxidant activity; tocopherol; chromanol

α-Tocopherol and its homologs are widely used as antioxidants additives in a variety of foods including edible oils and oil emulsions. However, these lipid-soluble types of vitamin E do not always provide effective protection against oxidative rancidity in food. Consequently, several studies have been carried out on the synthesis of α-tocopherol derivatives to improve the antioxidative efficiency of vitamin E. We have previously enzymatically synthesized a novel phosphatidyl derivative of vitamin E, i.e., 1,2-diacetyl-sn-glycero-3-phospho-2'-hydroxyethyl-2',5',7',8'-tetramethyl-6'-hydroxychroman (phosphatidylchromanol; PCh), by applying the transphosphatidylation activity of phospholipase D from Streptomyces lyicus. This vitamin E derivative was shown to possess higher affinity to phospholipid membranes and higher activity in improving the oxidative stability of lard than α-tocopherol. On the other hand, water-soluble vitamin E derivatives are likely to be more applicable for a wide range of water-containing foods and biological systems. In this present work, we attempt the synthesis of a water-soluble vitamin E derivative from PCh by phospholipase-catalyzed hydrolysis (Fig. 1).

PCh was prepared from dimyristoyl phosphatidylcholine (Sigma Chemical Co., St. Louis, MO, U.S.A.) and

Fig. 1. Molecular Structures of Vitamin E [α-Tocopherol], Phosphatidylchromanol [PCh, 2-(2',5',7',8'-Tetramethyl-6'-hydroxychromanyl)ethylphosphate], Toc-Et [2,5,7,8-Tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman] and Chromanol Phosphate [Ch-P, 2-(2',5',7',8'-Tetramethyl-6'-hydroxychromanyl)ethylphosphate].

2,5,7,8-tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman (Toc-Et; Kuraray Co., Kurashiki, Japan) by phospholipase D in a biphasic system according to the method described previously. Lyophilized powder of PCh (2.0 mg) was dispersed into 3.0 ml of a Tris-HCl buffer (10 mM, pH 7.4) by ultrasonic treatment with a sonifier (model W-380, Heat Systems; Farmingdale, NY, U.S.A.) for 5 min. Phospholipase C from Bacillus cereus (grade 1, 600 U; Boehringer, Mannheim, Germany) and diethyl ether (0.5 ml) were then added to the dispersion. The mixture was incubated at 37°C while vigorously shaking for 60 hrs. After this incubation, the reaction mixture was centrifuged at 3,500 rpm for 5 min. The aqueous phase was then taken out, washed twice with 2.0 ml of diethyl ether, adjusted to pH 2.5 by adding phosphoric acid, and extracted with 2.0 ml of ethyl acetate three times. The combined ethyl acetate layers were concentrated under a nitrogen stream, and the phosphorous content was determined by the method of Bartlett.

We selected phospholipase C from Bacillus cereus for the hydrolysis of PCh because this enzyme has been reported to hydrolyze a wide range of phospholipids.
It is also known that this enzyme has a marked preference for hydrolyzing a substrate at the lipid-water interface with the optimal pH value at around neutrality.\(^1\) The enzymatic reaction reached a plateau after 24 hrs of incubation, and the product of PCH was obtained in a yield of approximately 25%. The product gave a single broad peak at 4.5 min by reverse-phase HPLC in an octadecane-bonded silica column (Inertsil, 46.5 × 150 mm, 5 μm; GL Sciences, Tokyo, Japan) with an eluting solvent of acetonitrile/water (60:40, v/v) containing 0.01% trifluoroacetic acid. The eluent was monitored by UV absorption at 290 nm as a flow rate of 1.0 ml/min. In addition, the product gave a single blue spot on a silica gel 60 TLC plate with the Ditterm reagent\(^2\) and a red spot with the ferric chloride/bathophenantroline reagent,\(^3\) indicating the presence of phosphorous and reducing activity. The UV spectrum of the product in methanol showed the absorption maximum at 290 nm similarly to α-tocopherol (ε = 2,800). Fast atom bombardment-mass spectrometry, using a Jeol JMS-SX102A instrument (Japan Electric Optic Laboratory Co., Tokyo, Japan) showed a molecular ion at m/z 330 (M\(^+\)); 49% in addition to other fragment ions at m/z 185 ([Glycerol+H]\(^+\); 100%), m/z 423 ([M+glycerol+H]\(^+\); 64%), m/z 233 ([M-H\(_2\)PO\(_4\)+H]\(^+\); 58.4%), m/z 661 ([2M+H]\(^+\); 37.8%) and m/z 165([M-C\(_6\)H\(_5\)PO\(_4\)+H]\(^+\); 24%). Table 1 shows the \(^1\)H-NMR spectral data for the hydrolysis product and Toc-Et measured with a Jeol GSX-270 FT-NMR spectrometer at 270 MHz. Proton signals from the chromanol moiety of the product were essentially the same as those of Toc-Et. Two triplet signals of the product at 1.86 ppm and 1.75 ppm were assigned to H-3 and H-14, respectively. These proton signals showed downfield shifts by 0.13 ppm and 0.40 ppm, respectively, in comparison with those of Toc-Et. These data indicated the presence of a phosphate ester bond on the hydroxy group at C-15 of Toc-Et. In addition, two triplet signals at 1.62 ppm and 1.82 ppm, assigned to H-14 of Toc-Et, corresponded to a triplet signal at 1.75 ppm of the product, also indicating the presence of a phosphate ester bond. No proton signals from a phosphate moiety were detected in the spectrum. These spectral data confirmed the product to be 2-(2',5',7',8'-tetramethyl-6'-hydroxychromanoyl) ethylphosphate (Ch-P).

The free-radical scavenging activity of Ch-P was evaluated by using the stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to the method of Blois.\(^5\) Comparing the scavenging activity to that of cysteine, one molecule of Ch-P reacted with 2.5 molecules of DPPH (relative reactivity: cysteine, 1.0; Ch-P, 2.5; and α-tocopherol, 2.5). Thus, the number of radicals to be trapped by Ch-P was the same as that by α-tocopherol. Next, the antioxidative effects of Ch-P, PCH and α-tocopherol were compared on an emulsified fish oil (antioxidant-free sardine oil, Tsukishima Food Industrial Co., Tokyo, Japan). The fish oil emulsion was prepared from fish oil (23 μmol) and 2.0 ml of a Tris-HCl buffer (50 mm, pH 7.4) containing 10 mM Triton X-100 according to the method described previously.\(^6\) Oxidation was started by adding 0.2 ml of the 50 mm Tris-HCl buffer (pH 7.4) containing 0.5 mM Fe(NO\(_3\))\(_3\) and 5 mM L-ascorbic acid to the emulsion (0.8 ml). The reaction was conducted at 37°C in the dark while continuously shaking. Aliquots of the reaction mixture were subjected to a thiobarbituric acid (TBA) assay\(^7\) as the reaction progressed. The fatty acid composition of the fish oil in the reaction mixture was determined by a gas chromatographic analysis after extracting the oil and transmethyllating by the method previously reported.\(^8\) Ch-P suppressed the accumulation of TBA-reactive substances more effectively than α-tocopherol or PCH (Fig. 2). The superiority of Ch-P over the other two compounds was confirmed by the rate of decrease of the polyunsaturated fatty acid moiety obtained by gas chromatographic analysis\(^9\) (Table 2). The decreased contents of eicosapentaenoic acid and docosahexaenoic acid were effectively suppressed by the addition of Ch-P. These phenomena demonstrate that Ch-P was an effective antioxidant for preventing the metal ion-catalyzed oxidation of the fish oil emulsion. Solubility in the aqueous phase of the emulsion may increase the ability of the chromanol moiety to trap the radicals responsible for lipid peroxidation.

In the last experiment, the antioxidative effect of Ch-
P was evaluated on a biological tissue homogenate. The whole brain was removed from a male Wistar rat (7 wk old), minced and washed with phosphate-buffered saline. The brain was homogenized with Polytron device in 6 volumes (w/v) of an ice-cold 0.1 M Tris-HCl buffer (pH 7.4) containing 0.135 M KCl. The homogenate was centrifuged at 3,500 rpm for 10 min at 4°C. A sample of the supernatant was removed and diluted with 4 volumes of the same buffer. The reaction mixture consisting of 980 μl of the diluted homogenate and 20 μl of the Ch-P solution in a 50 mM Tris-HCl buffer or an ethanol solution of PCh or α-tocopherol was incubated at 37°C. A TBA assay was carried out after 4 hrs of incubation. Figure 3 shows the concentration of three compounds required for 50% inhibition of TBA-reactive substance accumulation; 0.19 μM, 0.74 μM and 3.84 μM for Ch-P, PCh and α-tocopherol, respectively. It is thus apparent that Ch-P was the most effective antioxidant among the three compounds toward brain homogenate autoxidation.

In conclusion, we synthesized a new phosphate ester of a vitamin E derivative with high solubility in the aqueous phase. Its antioxidative activity seems to be comparable or superior to that of vitamin E in an aqueous suspension. One of the authors has already reported the synthesis of a water-soluble glucosyl derivative of vitamin E. However, the unique characteristic of the phosphate group in Ch-P also warrants its potential application as a water-soluble antioxidant for food and biological systems.

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