Quenching of Peroxynitrite by Lycopene in Vitro

Tadashi Yokota, Teturou Ohtake, Hajime Ishikawa,[†] Takahiro Inakuma,[††] Yukio Ishiguro,[††] Junji Terao,[†††] Akihiko Nagao,[††††] and Hideo Etoh*

Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529

[†]*Living Environmental Division, Aichi Environmental Research Center, 7-6 Nagare, Tsuji-machi, Kita, Nagoya 462-0032*

[††]*Kagome Co., Ltd., 17 Nishitomiyama, Nishinasuno-cho, Nasu-gun, Tochigi 329-2762*

[†††]*Faculty of Medicine, Tokushima University, 3-18-15 Kuramoto-cho, Tokushima, 770-8503*

[††††]*National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, 2-1-12 Kannnondai, Tsukuba, 305-8642*

(Received October 23, 2003; CL-031006)

We investigated in vitro the reactivity of lycopene toward peroxynitrite and identified the reaction products after scavenging peroxynitrite. On the basis of these studies, we propose a mechanism for peroxynitrite scavenging action of lycopene.

Lycopene is a naturally occurring carotenoid, which owes its ruby-red color to the presence of an extensively conjugated polyene structure. Such a structure renders lycopene highly susceptible to oxidation. Literature indicates that lycopene is an effective scavenger for reactive oxygen species and protects against oxygen-mediated cytotoxicity and genotoxicity by scavenging singlet oxygen and other reactive oxygen species.[1,2]

Peroxynitrite is a strong oxidant produced in vivo as a product of reaction of superoxide anion and nitric oxide. Peroxynitrite and its decomposition products are reported to induce both DNA strand scission and protein modification by nitration, hydroxylation, and membrane lipid peroxidation in low density lipoprotein (LDL).[3,4] Recently, it was reported that carotenoids readily reacted with peroxynitrite. The reactivity of β-carotene toward NO, NO_2, peroxynitrite, and 3-morpholinosydononimine (SIN-1) that generates peroxynitrite was examined and was shown to possess strong scavenging activity for all the three above-mentioned species.[5] However, the peroxynitrite scavenging mechanism of carotenoids is unknown in vivo or in vitro. We investigated in vitro the reactivity of lycopene with peroxynitrite and isolated the reaction products after scavenging peroxynitrite.[6]

To a solution of lycopene (500 mg, 0.93 mmol) and TFA (20 μL) in 200 mL of THF was added 4 mL of 50 mM peroxynitrite solution. The reaction mixture was stirred for 1 min at room temperature in darkness. The reaction mixture was then successively washed saturated aqueous $NaHCO_3$ and distilled water, dried over anhydrous Na_2SO_4, filtered and concentrated. The resulting solution was applied to HPLC on C-30 to analyze the products.

Some peaks of *Rt* 17–32 min appeared after addition of methanol that recovered to all-*trans*-lycopene (30 mg, Figure 1). It is important to assign these peaks to understand reaction mechanism of lycopene with peroxynitrite. Several oxidative products of *Rt* 17–32 min exhibited about 20–40 nm hypsochromic shift in their UV–vis spectrum when compared to all-*trans*-lycopene, indicating the presence of decreased conjugation. Compound **I** (3 mg, peak 7) in the HPLC chromatogram was identified as (2*R**, 5*S**, 6*R**)-1-methoxy-2,6-cyclolycopene-5-ol by comparing its spectroscopic data with those from tomato puree.[7] The molecular weight of **II**[8] (3 mg, peak 8) and **III**[9] (2 mg, peak 9) was established to be 598 by EI–MS. ^{13}C NMR of compound **II** showed 21 signals, which indicated cleavage of lycopene into two equal halves. ^1H NMR of compound **II** indicated the presence of methoxy groups at δ 3.23. Coupling constant values (15.4 Hz) $J_{H(6)\text{-}H(7)}$ indicated that the double bond at C5–C6 changed to C6–C7. The HSQC, DEPT, and HMBC spectra of compound **II** showed that methoxy group addition occurred at 5,5′ position of lycopene, to yield 5,5′-dimethoxy-lycopene. ^1H NMR of compound **III** indicated two methoxy groups at δ 3.10 and 3.11. The ^1H NMR and ^{13}C NMR chemical shifts of compound **III** are similar to those of 2,6-cyclolycopene-1,5-diol[10,11] and compound **I**.[7] The HSQC and DEPT spectra of compound **III** showed two quaternary oxycarbons at δ 76.76 (C-1) and δ 86.88 (C-5), two methine carbons at δ 51.42 (C-2) and δ 57.14 (C-6), and two methylene carbons at δ 24.29 (C-3) and δ 33.91 (C-4), two methoxy carbons at δ 48.99 (5-OMe) and δ 49.55 (1-OMe). The correlations observed in the ^1H–^1H COSY spectrum indicated a partial structure –C(4)H_2–C(3)H_2–C(2)H–C(6)H–C(7)H=C(8)H–. HMBC spectra showed that the terminal group of this compound has a dimethoxy iridane skeleton. Thus compound **III** was found to be a (2*R**, 5*S**, 6*R**)-1,5-dimethoxy-2,6-cyclolycopene.

The peaks of *Rt* 8–12 min were assigned by comparison of retention time and UV–vis spectra with those of the reference cleavage products prepared by ozonolysis of lycopene.[12] We identified acycloretinal (peak 1), apo-14′-lycopenal (peak 2), apo-12′-lycopenal (peak 3), apo-10′-lycopenal (peak 4), apo-

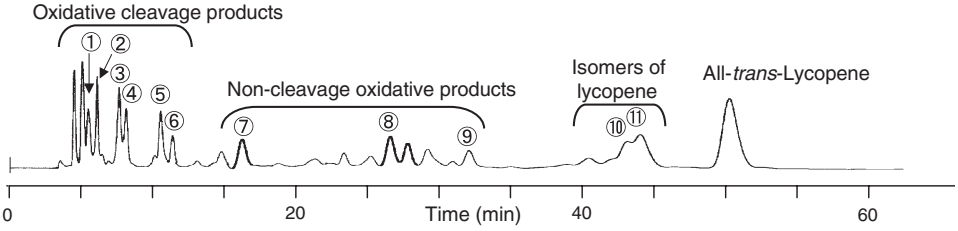

Figure 1. HPLC Analysis of Peroxynitrite Reaction Products of Lycopene. Column: Develosil C30-UG-5 ϕ4.6 × 250 mm, Mobile phase: acetonitrile-methanol-*n*-hexane-dichloromethane-DIPEA = 85: 10: 2.5: 2.5: 0.1, Detection: 350 nm–550 nm, Flow rate: 1.0 mL/1 min., Column temp.: 40 °C.

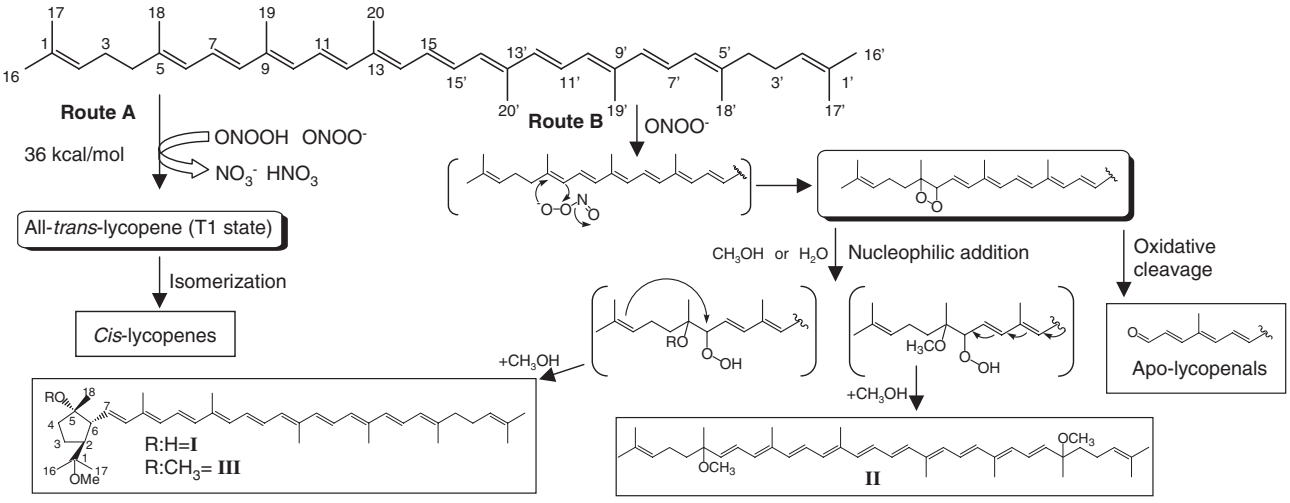

Figure 2. Proposed Mechanisms for Reaction of Lycopene with Peroxynitrite.

8′-lycopenal (peak 5), and apo-6′-lycopenal (peak 6). These peaks of *Rt* 43–50 min exhibited 8 nm hypsochromic shift and had absorption at about 360 nm in their UV–vis spectrum. This absorption was called *cis*-peak.[13] The compounds of peak 10 and 11 were identified as 13- and 9-*cis*-lycopene by comparing UV and ^{13}C NMR data with those from the literature.[14]

On the basis of above reaction products, we suggest the reaction mechanism of lycopene with peroxynitrite (Figure 2). Route A, all-*trans*-lycopene accepts energy from peroxynitrite and goes to an excited state (biradical), and while returning to the ground state produces *cis*-isomers. Recently, density functional theory (DFT) approach of the singlet oxygen–carotenoid interaction has been suggested. This study had shown that the $S_0 \rightarrow T_1$ vertical excitation energy was 26.1 kcal mol^{-1} computed for the longer 11 conjugated polyene.[15] It was suggested that the terminal oxygen group of peroxynitrite attacked nitrogen to form nitric acid, this arrangement of peroxynitrite anion to nitrate is extremely favorable, releasing 36 kcal mol^{-1} of energy at pH 7.0.[3] We attempted to elucidate the structure of intermediate of compounds **I**, **II**, and **III**, and apo-lycopenals from lycopene. A comparison with *m*CPBA oxidation products indicated that the reaction of peroxynitrite with lycopene was different from the reported oxidation mechanism. Production of lycopene-1,2-epoxide and lycopene-5,6-epoxide, the reported reaction intermediates of *m*CPBA and H_2O_2 oxidation,[11] were not confirmed. On the other hand, compounds **I**, **II**, and **III** were observed in the reaction of lycopene with singlet oxygene[2] in $CHCl_3$ under coexistence of methanol and the chromatogram of other peaks showed also the same retention time and UV spectra. We suggest that the reaction intermediate was dioxetane that produced compound **I**, **II**, and **III** and apo-lycopenals. Route B, all-*trans*-lycopene directly react with peroxynitrite to produce dioxetan that cleaves to apo-lycopenals or undergoes methanolysis to yield methoxy-lycopene. Reaction of lycopene with peroxynitrite in vivo activity is now in progress.

The authors thank Dr. Shoske Yamamura (professor emeritus of Keio University) for several discussions and comments.

References and Notes

1 P. DiMacio, S. Kaiser, and H. Sies, *Arch. Biochem. Biophys.*, **274**, 532 (1989).
2 Y. Lu, H. Etoh, N. Watanabe, K. Ina, N. Ukai, S. Oshima, F. Ojima, H. Sakamoto, and Y. Ishiguro, *Biosci. Biotechnol. Biochem.*, **59**, 2153 (1995).
3 J. P. Crow, Spruell, C. Chen, J. Gunn, C. Harry, H. Ischiropoulos, M. Tsai, C. D. Smith, R. Radi, W. H. Coppenol, and J. S. Beckman, *Free Radical Biol. Med.*, **16**, 331 (1994).
4 A. Gow, D. Duran, S. R. Thom, and H. Ischiropoulos, *Arch. Biochem. Biophys.*, **333**, 42 (1996).
5 K. Kikugawa, K. Hiramoto, S. Tomiyama, and Y. Asano, *FEBS Lett.*, **404**, 175 (1997).
6 T. Yokota, T. Ohtake, R. Suzuki, H. Etoh, S. Oshima, T. Inakuma, and Y. Ishiguro, 45th Symposium on The Chemistry of Natural Products, Kyoto, 2003, Abstr., No. 449.
7 T. Yokota, H. Etoh, S. Oshima, K. Hayakawa, and Y. Ishiguro, *Biosci. Biotechnol. Biochem.*, **67**, 2644 (2003).
8 Compound **II**: EIMS *m*/*z*: 598 (M^+); UV λ_{max}(hexane)nm: 406, 430, 463; ^{1}H NMR δ($CDCl_3$): 1.26(3H, s), 1.28(3H, s), 1.60(3H, s), 1.80(6H, s, H-19 and 20), 1.90(4H, m, H-3 and 4), 3.23(3H, s, OMe), 5.10(2H, t, J = 7.3 Hz, H-2), 5.68(3H, d, J = 15.3 Hz, H-6), 6.15(1H, d, J = 11.0 Hz, H-8), 6.20(1H, d, J = 11.3 Hz, H-12), 6.35(1H, d, J = 15.0 Hz, H-10), 6.35(1H, m, H-14), 6.50(1H, dd, J = 11.0, 15.3 Hz, H-7), 6.67(1H, dd, J = 11.3, 15.0 Hz, H-11), 6.67(1H, m, H-15); ^{13}C NMR δ ($CDCl_3$): 12.79(C-19), 12.82(C-20), 17.66(C-17), 22.42(C-3), 22.50(C-18), 25.69(C-16), 39.73(C-4), 50.13(OMe), 75.50(C-5), 124.44(C-2), 124.95(C-11), 124.96(15), 126.52(C-7), 129.06(C-1), 131.51(C-8), 132.45(C-12), 135.65(C-13), 135.80(C-9), 137.33(C-14), 137.89(C-10), 139.37(C-6).
9 Compound **III**: EIMS *m*/*z*: 598 (M^+); UV λ_{max}(hexane)nm: 430, 455, 487; ^{1}H NMR δ($CDCl_3$): 1.07(3H, s, H-16), 1.08(3H, s, H-17), 1.14(3H, s, H-18), 1.31(1H, m, H-4a), 1.61(3H, s, H-17′), 1.65(1H, m, H-3a), 1.69(3H, s, H-16′), 1.76(1H, m, H-3b), 1.82(3H, s, H-18′), 1.94(3H, s, H-19), 1.97(9H, s, H-19′, 20 and 20′), 2.00(1H, m, H-4b), 2.11(4H, m H-3′ and 4′), 2.17(1H, dd, J = 9.2, 1.5 Hz H-6), 2.36(1H, m, H-2), 3.13(3H, s, 5-OMe), 3.14(1-OMe), 5.11(1H, m, H-2′), 5.76(1H, dd, J = 9.6, 15.6 Hz, H-7), 5.95(1H, d, J = 11.0 Hz, H-6′), 6.07(1H, d, J = 15.6 Hz, H-8), 6.14(1H, d, J = 11.5 Hz, H-10), 6.18(1H, d, J = 11.5 Hz, H-10′), 6.23(2H, m, H-14 and 14′), 6.25(1H, d, J = 15.0 Hz, H-8′), 6.33(2H, d, J = 14.6 Hz, H-12 and 12′), 6.49(1H, dd, J = 11.0, 15.0 Hz, H-7′), 6.63(2H, m, H-15 and 15′), 6.64(2H, dd, J = 11.5, 14.6 Hz, H-11, 11′); ^{13}C NMR δ($CDCl_3$): 12.79(C-20 and 20′), 12.91(C-19′), 13.22(C-19), 16.97(C-18′), 17.71(C-17′), 20.19(C-18), 23.02(C-16), 24.09(C-17), 24.29(C-3), 25.71(C-16′), 26.71(C-3′), 33.91(C-4), 40.25(C-4′), 48.99(1-OMe), 49.45(5-OMe), 51.42(C-2), 57.14(C-6), 76.76(C-1), 86.88(C-5), 123.91(C-2′), 124.66(C-7′), 125.10(C-11 and 11′), 125.75(C-6′), 129.95(C-15′), 129.96(C-15), 130.09(C-10), 131.48(C-10′), 131.56(C-1′), 131.80(C-7), 132.25(C-8′), 132.64(C-14, 14′), 136.04(C-8), 136.16(C-9), 136.24(C-9′), 136.56(C-13), 136.67(C-13′), 137.34(C-12), 137.38(C-12′), 139.48(C-5′).
10 T. Yokota, H. Etoh, N. Ukai, S. Oshima, H. Sakamoto, and Y. Ishiguro, *Biosci. Biotechnol. Biochem.*, **61**, 549 (1997).
11 F. Khachik, A. Steck, U. A. Niggli, and H. Pfander, *J. Agric. Food. Chem.*, **46**, 4874 (1998).
12 S.-J. Kim, E. Nara, H. Kobayashi, J. Terao, and A. Nagao, *Lipids*, **36**, 191 (2001).
13 W. Stahl, W. Schwarz, A. R. Sundquist, and H. Sies, *Arch. Biochem. Biophys.*, **294**, 173 (1992).
14 A. Z. Mercadante, A. Steck, and H. Pfander, *J. Agric. Food. Chem.*, **47**, 145 (1999).
15 M. Garavelli, F. Bernardi, M. Olivucci, and M. A. Robb, *J. Am. Chem. Soc.*, **120**, 10210 (1998).

Published on the web (Advance View) December 20, 2003; DOI 10.1246/cl.2004.80