Abstract: Mono- (5Z)-, (9Z)-, and (13Z)-lycopenes are found in food containing processed tomato products, while tetra-Z-(7Z, 9Z, 7′Z, 9′Z)-lycopene (prolycopene) is found in tangerine-strain tomatoes. We prepared pure mono-Z-lycopenes from all-E-lycopene via chemical reaction (heating in CH₂Cl₂ at 80°C for 1 h) followed by purification using preparative silica gel HPLC, while prolycopene was isolated from tangerine tomatoes by partitioning with n-hexane and 90% MeOH followed by silica gel column chromatography. A simple method of distinguishing the mono-Z-lycopenes using the 13C NMR chemical shifts of their Z-methyl carbons is proposed. Additionally, the 1O₂ quenching and 3T3-L1 cell differentiation activities of the compounds were then compared with all-E-lycopene for the first time. All the evaluated Z-isomers showed 1O₂ quenching activities that were equal to or slightly lower than that of all-E-lycopene, with the IC₅₀ values for the 1O₂ quenching activities of (all-E)-, (5Z)-, (9Z)-, (13Z)-, and (7Z, 9Z, 7′Z, 9′Z)-lycopene being 4.4±0.36, 4.0±1.44, 5.3±1.08, 6.9±1.67, and 8.7±0.34 μM, respectively. The mouse 3T3-L1 cell differentiation activities followed the order: (all-E) > (9Z) > (5Z) ≈ (9Z) ≈ (13Z) ≈ (7Z, 9Z, 7′Z, 9′Z).

Key words:

1 Introduction

All-E-lycopene (AE-L, Fig. 1) is a principal carotenoid contained in ripe tomato (Solanum lycopersicum L.). AE-L has been claimed to possess cardioprotective effects⁵ due to its antioxidant activity², as well as anti-diabetic effects¹,⁴ due to its antioxidant³ and PPARγ agonistic activities⁶. Some Z-isomers of AE-L have also been reported to be present in foods in previous studies; for example, mono-(5Z)-, (9Z)-, and (13Z)-lycopenes (5Z-L, 9Z-L, and 13Z-L, Fig. 1) have been reported to be produced during the cooking of ripe tomatoes, and are claimed to be present in tomato puree, ketchup, and soup at levels of 10–30%.⁷. The tetra-Z-(7Z, 9Z, 7′Z, 9′Z)-lycopene (prolycopene, Fig. 1) has also been reported to exist in tangerine-strain tomatoes due to the lack of carotenoid isomerase (CRTISO)⁸, which converts prolycopene into AE-L⁹. Unlike AE-L, few of these Z-lycopenes have been reported to show biological activities, with the exception of the radical-quenching activities of 5Z-L, 9Z-L, and 13Z-L⁹ and the PPARγ agonistic activity of 5Z-L¹⁰. This is due to the difficulty of preparing enough of the pure compounds for bioactivity tests.

Studies on the bioavailabilities and pharmacological activities of Z-isomers of β-carotene were carried out in the 1990s and 2000s¹⁰⁻¹⁵, as all-E-β-carotene can be synthesized at a low cost while Z-isomers of β-carotene are readily available from Dunaliella sp.¹⁰,¹¹. These studies demonstrated that Z-isomers of β-carotene exhibited lower bioavailability than all-E-β-carotene and provitamin A (retinol and retinal), and that the antioxidant activities of the Z-isomers of β-carotene were also equal to or lower than that of all-E-β-carotene¹⁰⁻¹⁵. As a result, research into the pharmacological activities of Z-isomers of carotenoids fell out of the limelight at that time. However, in recent years several studies have reported that Z-isomers of lycopene and astaxanthin have higher bioavailability than their respective all-E-isomers¹⁶⁻¹⁸. For example, in 2015 Cooperstone et al. reported that a human oral administration test showed that Z-isomer-rich tangerine tomato juice was over 8 times more bioavailable than AE-L-rich red tomato juice¹⁷. Furthermore, Z-isomers of astaxanthin and fucoxanthin have been shown to possess greater anticancer and anti-inflammatory activities than their respective all-E-isomers¹⁹,²⁰. This research has led to increasing interest in the pharmacological activities of Z-isomers of carotenoids in recent years.

In this report, we prepared 5Z-L, 9Z-L, and 13Z-L from...
lycopene via chemical reactions and purified them, and purified prolycopene from tangerine-strain tomatoes. We evaluated the $^1$O$_2$ quenching activities and 3T3-L1 cell differentiation activities of 5Z-L, 9Z-L, 13Z-L, and prolycopene in comparison with AE-L for the first time. In addition, we report a simple method of distinguishing 5Z-L and 9Z-L using the characteristic chemical shifts of the Z-methyl $^{13}$C NMR peaks.

2 Material and Methods

2.1 Chemicals

CH$_2$Cl$_2$, n-hexane, ethanol, MeOH, and 2-propanol were of analytical grade and purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Benzene of analytical grade and N,N-diisopropylethylamine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). DMEM and PBS (−) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Spectroscopic analysis

NMR spectra were measured in CDCl$_3$ with an AVANCE400 instrument (Bruker BioSpin, Karlsruhe, Germany), using the residual solvent peak as an internal standard ($\delta_c$ 77.0, $\delta_h$ 7.26 ppm).

2.3 Extraction and purification of AE-L

CH$_2$Cl$_2$ (800 mL) was added to 400 g tomato paste (Kagome Co., Ltd.; Tokyo, Japan) in a 2 L Erlenmeyer flask, and the mixture was stirred for 60 min in darkness at room temperature. The layers were separated using a separatory funnel, and repetitive extraction was performed on the suspension formed in the aqueous layer twice using the same volume of CH$_2$Cl$_2$. After drying over Na$_2$SO$_4$ for 30 min, the combined CH$_2$Cl$_2$ layers (2.4 L) were concentrated to dryness in vacuo to afford crude lycopene extract (1.50 g). The extract was dissolved in 5 mL benzene at 60°C using sonication and recrystallized at 4°C overnight in the dark. The crystals were collected by suction filtration on a Kiriyama funnel (No. 5C filter paper) and rinsed with 100 mL of acetone. The resulting red crystalline powder on the filter paper was collected using a medicine spoon into a 200 mL eggplant flask and dried in vacuo to afford pure AE-L (0.50 g).

2.4 Thermal isomerization of AE-L

Thermal isomerization of AE-L was performed according to a previously reported method$^{21}$ with some modifications. In brief, purified AE-L (100 mg) was dissolved in 100 mL CH$_2$Cl$_2$ (1 mg/mL) and transferred into a 100 mL pressure-resistant stainless-steel vessel (TVS-1; Taiatsu Techno Corp., Saitama, Japan). The head space was purged with N$_2$ gas and the vessel was immediately closed tightly to minimize oxygen exposure and placed in a water bath at 80°C for 1 h. After this, the vessel was removed from the water bath and cooled to room temperature. The CH$_2$Cl$_2$ solution containing the mono-Z-isomers (5Z-L, 9Z-L, and 13Z-L) was then moved to a 200 mL eggplant flask and
2.5 Purification of 5Z-L, 9Z-L, and 13Z-L

The mixture of mono-Z-lycopenes (5Z-L, 9Z-L, and 13Z-L) obtained by thermal isomerization was purified by preparative HPLC (column: three Nucleosil 300-5 columns (10 x 250 mm, GL Science Inc., Tokyo, Japan) in tandem; solvent: n-hexane containing N,N-diisopropylethylamine (1000:1, v/v); detector: photodiode array (250–700 nm)). Compounds 13Z-L, 9Z-L, and 5Z-L eluted at 50.0, 61.0, and 72.5 min, respectively (AE-L eluted at 70.5 min) (Fig. 2). The identification and purity of each Z-isomer was checked by 1H and 13C NMR spectroscopies (Figs. S1–S6). Yields of pure 13Z-L, 9Z-L, and 5Z-L from 100 mg of AE-L were 1.3, 2.0, and 2.6 mg, respectively. Purified 13Z-L, 9Z-L, and 5Z-L were concentrated to dryness in light-tight glass tubes (0.5 mg/tube) and kept in −80°C freezer under N2 atmosphere.

2.6 Purification of tetra-Z-(7Z, 9Z, 7’ Z, 9’ Z)-lycopene

Ripe tangerine-strain tomatoes were purchased (Momotaro gold, 1 kg; Toyota City, Aichi Prefecture, Japan) and crushed with a mixer for 1 min. The obtained paste (800 mL) was added to an equal volume of acetone and stirred for 10 min. The solution was filtered under reduced pressure, and the resulting material on the filter paper was recovered and extracted twice with CH2Cl2 (500 mL). The resulting CH2Cl2 solution (1 L) was concentrated to a smaller volume in vacuo, and partitioned with n-hexane (300 mL) and MeOH (90% v/v, aq.) (300 mL) in a separating funnel. The n-hexane layer was concentrated to dryness in vacuo, and the resulting material was purified using silica gel column chromatography (2 cm x 20 cm, FL60D (CHROMATOEX, Japan)) using n-hexane/CH2Cl2 (20:1, v/v) as an eluent. The fractions containing a yellow compound (Rf = 0.2 as determined using thin layer chromatography on silica gel (Merck; solvent = n-hexane/CH2Cl2 (20:1, v/v)) was collected to afford pure prolycopene (2.5 mg). The identity and purity of prolycopene were checked using 1H and 13C NMR spectroscopies (Figs. S7 and S8). Purified prolycopene were concentrated to dryness in light-tight glass tubes (0.5 mg/tube) and kept in −80°C freezer under N2 atmosphere.

2.7 O2 quenching experiment

Methylene Blue (80 µL, 25 µM in ethanol) and linoleic acid (100 µL, 0.24 M in ethanol) were added to 5 mL glass test tubes with and without 40 µL of carotenoid (final concentration, 1–100 µM in ethanol). The tubes were mixed well, and then illuminated at 7,000 lux and 22°C for 3 h in a Styrofoam box, after which 50 µL of the reaction mixture was removed and diluted to 1.5 mL with ethanol. OD235 was then measured to estimate the levels of conjugated dienes formed in the reaction (Fig. 2). OD235 in the absence of the carotenoid was also measured as a negative control (no O2 quenching activity), and the O2 quenching activity of the carotenoid was then calculated relative to this reference value. The activity was determined as the IC50 value, representing the concentration at which 50% inhibition was observed.

2.8 3T3-L1 adipogenic-differentiation bioassay

The 3T3-L1 cell line was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum (Sigma-Aldrich, St. Louis, USA) and antibiotics (62.5 µg/mL penicillin and 100 µg/mL streptomycin). The cells were seeded in collagen-coated 12-well plates at 40,000 cells/well (1 mL) and grown to confluence for 2 days. Differentiation was initiated 2 days after cells reached confluence by changing the medium to a differentiation cocktail (3 µg/mL insulin in DMEM containing 10% fetal bovine serum and antibiotics) with or without test compounds dissolved in THF contain-
Y. Sakemi, K. Sato, K. Hara et al.

J. Oleo Sci.

ing 0.03% (v/v) BHT (2,6-Di-t-butyl-4-methylphenol) (THF solution was added to the medium at a ratio of 0.1%). Thereafter, the cells were grown in the differentiation cocktail for 9 days. Oil Red O (ORO) staining was then performed as previously reported. Briefly, the differentiated 3T3-L1 cells were fixed with 4% (v/v) formalin for 1h and washed twice with PBS (-). The cells were then rinsed with 60% isopropanol for 5min, followed by a 10 min incubation with freshly prepared ORO working solution (0.5 g of ORO in 100 mL of isopropanol was added to 67 mL of water and filtered through a 0.45 µm filter). After washing twice with PBS (-) (1 mL), ORO was extracted from the fixed cells with 100% isopropanol (1 mL) and the OD₅₆₀ values of the isopropanol-based extracts (200 µL) were read in a 96-well microplate reader. The relative lipid content of each well is presented as a percentage of the value of the corresponding control (set at 100%)

2.9 Statistical analysis

Data obtained in 3T3-L1 adipogenic-differentiation bioassay were analyzed using one-way analysis of variance between subjects, and post-hoc comparisons were made using Tukey’s honestly significant difference test. In all cases, statistical significance was set at p < 0.05.

Comparisons of 1 O₂ quenching activities of lycopene isomers were made with Student’s t test.

3 Results and Discussion

3.1 Characterization of lycopene isomers

5Z-L, 9Z-L, 13Z-L, and prolycopene were successfully purified from thermally treated lycopene and tangerine-strain tomato. These isomers were analyzed by ¹H and ¹³C NMR spectroscopies (Figs. S1–S6), and the observed ¹H and ¹³C chemical shifts of the isomers were in good accordance with those previously reported. ¹³C NMR data of 5Z-L, 9Z-L, 13Z-L, and prolycopene is listed in Table 1. In addition, we also listed UV/VIS data of them as reference (Table S-1).

When we compared the ¹³C NMR data of the mono-Z-lycopenes, we found a simple method of distinguishing 5Z-L, 9Z-L, and 13Z-L using the ¹³C NMR shifts of the Z-methyl signals observed at δ₂: 20–25, as no other signals were observed in this area. We could identify 5Z-L, 9Z-L, 13Z-L unambiguously using the ¹³C chemical shifts of C-18 in 5Z-L (δ₁ 24.13), C-19 in 9Z-L (δ₁ 20.79), and C-20 in 13Z-L (δ₁ 20.69).

3.2 O₂ quenching activities of lycopene isomers

Several studies have evaluated the antioxidant activities of lycopene isomers, and some assays have indicated that the activities of these isomers differ. However, such evaluations investigated the scavenging activities for peroxyl radicals and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cations, while there have been no evaluations of their O₂⁻ quenching activity, for which carotenoids have proved highly effective. The O₂⁻ quenching activities of 5Z-L, 9Z-L, 13Z-L, and prolycopene were examined and compared with that of AE-L, as shown in Table 2. 5Z-L showed almost the same degree of the O₂⁻ quenching activity as AE-L, whereas the activities of 9Z-L, 13Z-L and prolycopene were slightly lower than that of AE-L. At present, it is therefore judged that Z-isomerization has little effect on the O₂⁻ quenching activity of AE-L.

Processed tomato products such as pasta sauce and pizza sauce contain large amounts of lycopene Z-isomers, primarily 5Z-L. In addition, 5Z-L and 9Z-L are the major Z-configurations found in animal bodies. For example, when AE-L- or 13Z-L-rich diets were orally administered to mice, 5Z-L and 9Z-L were detected as the major isomers in the liver in both cases. Therefore, the information that 5Z-L and 9Z-L exert the same degree of O₂⁻ quenching as AE-L is very important for the fields of cookery science and food nutrition.

Previous studies on the radical scavenging activities of 5Z-L, 9Z-L, 13Z-L, and AE-L showed that they all had similar activities in the ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assays, while 5Z-L, 9Z-L, and prolycopene showed higher antioxidant activity than AE-L in the LPSC assay. Moreover, 5Z-L exhibited greater activity than 9Z-L, 13Z-L, and AE-L for the inhibition of the MbFe³⁺ lipid peroxidation of linoleic acid. Our results indicate that structural differences may be required to improve singlet oxygen scavenging and radical species scavenging activities.

3.3 3T3-L1 cell differentiation activity of lycopene isomers

Peroxisome proliferator-activated receptor (PPAR)γ is a ligand-activated transcription factor of the nuclear hormone receptor superfamily. Activation of PPARγ enhances adipocyte differentiation and, thus, causes insulin sensitization and improved glucose metabolism. Currently, PPARγ agonists such as rosiglitazone are used clinically to treat patients with T2DM. Thus, PPARγ agonists play critical regulatory roles in energy homeostasis and metabolic functions. 5Z-L was reported to possess superior transactivation activity of PPARγ to AE-L when studied using a reporter gene assay and 3T3-L1 cells. To investigate whether 5Z-L, 9Z-L, 13Z-L, prolycopene, and AE-L were capable of PPARγ signal activation in cells, we performed 3T3-L1 differentiation studies for each of them. The results are shown in Fig. 3. All Z-isomers (5Z-L, 9Z-L, 13Z-L, and prolycopene) showed slightly weaker differentiation activities than AE-L. Thus, we demonstrated that AE-L and its Z-isomers possess PPARγ agonistic activities at the cellular level, and that AE-L shows more potent activity than its Z-isomers.

J. Oleo Sci.
Table 1. $^{13}$C NMR data of all-$E$-lycopene (AE-L), (5Z)-lycopene (5Z-L), (9Z)-lycopene (9Z-L), (13Z)-lycopene (13Z-L), and prolycopene in CDCl$_3$.

|          | AE-L   | 5Z-L   | 9Z-L   | 13Z-L  | prolycopene |
|----------|--------|--------|--------|--------|-------------|
| C-1      | 131.75 | 131.99 | 131.78 | 131.76 | 131.71      |
| C-1'     | 131.77 | 131.73 | 131.72 | 131.74 | 131.71      |
| C-2      | 123.95 | 123.99 | 123.95 | 123.95 | 123.96      |
| C-2'     | 123.95 | 123.96 | 123.88 | 123.95 | 123.96      |
| C-3      | 26.69  | 26.86  | 26.68  | 26.68  | 26.69       |
| C-3'     | 26.69  | 26.69  | 26.68  | 26.68  | 26.69       |
| C-4      | 40.24  | 32.80  | 40.26  | 40.23  | 40.32       |
| C-4'     | 40.24  | 40.23  | 40.22  | 40.23  | 40.32       |
| C-5      | 139.50 | 139.48 | 139.45 | 139.73 | 140.89      |
| C-5'     | 139.50 | 139.70 | 140.40 | 139.43 | 140.89      |
| C-6      | 125.73 | 126.53 | 125.72 | 125.68 | 122.48      |
| C-6'     | 125.73 | 125.73 | 125.79 | 125.72 | 122.48      |
| C-7      | 124.80 | 124.67 | 126.30 | 125.00 | 126.16      |
| C-7'     | 124.80 | 124.80 | 124.77 | 124.74 | 126.16      |
| C-8      | 135.40 | 135.12 | 127.23 | 135.30 | 125.92      |
| C-8'     | 135.40 | 135.40 | 135.40 | 135.41 | 125.92      |
| C-9      | 136.17 | 136.15 | 134.60 | 136.36 | 135.51      |
| C-9'     | 136.17 | 136.15 | 136.11 | 136.06 | 135.51      |
| C-10     | 131.55 | 131.55 | 129.94 | 131.48 | 129.83      |
| C-10'    | 131.55 | 131.47 | 131.54 | 131.54 | 129.83      |
| C-11     | 125.16 | 125.14 | 123.89 | 126.43 | 126.21      |
| C-11'    | 124.16 | 125.14 | 125.09 | 125.09 | 126.21      |
| C-12     | 137.36 | 137.31$^b$ | 136.64 | 129.15 | 136.09      |
| C-12'    | 137.36 | 137.35$^b$ | 137.36 | 137.38 | 136.09      |
| C-13     | 136.55 | 136.54 | 136.44 | 134.90 | 136.35      |
| C-13'    | 136.55 | 136.54 | 136.47 | 136.86 | 136.35      |
| C-14     | 132.64 | 132.64$^e$ | 132.42 | 132.93 | 131.96      |
| C-14'    | 132.64 | 132.61$^i$ | 132.62 | 132.47 | 131.96      |
| C-15     | 130.08 | 130.07 | 129.94 | 128.78 | 129.72$^j$ |
| C-15'    | 130.08 | 130.07 | 130.06 | 129.23 | 129.72$^j$ |
| C-16     | 25.70  | 25.70$^d$ | 25.69  | 25.69  | 25.64       |
| C-16'    | 25.70  | 25.69$^d$ | 25.69  | 25.69  | 25.64       |
| C-17     | 17.70  | 17.64  | 17.69  | 17.69  | 17.61       |
| C-17'    | 17.70  | 17.69  | 17.69  | 17.69  | 17.61       |
| C-18     | 16.96  | 24.13  | 16.98  | 16.95  | 16.59       |
| C-18'    | 16.96  | 16.95  | 16.95  | 16.95  | 16.59       |
| C-19     | 12.90  | 12.89  | 20.79  | 12.93  | 24.71       |
| C-19'    | 12.90  | 12.89  | 12.88  | 12.89  | 24.71       |
| C-20     | 12.80  | 12.79  | 12.88  | 20.69  | 12.68       |
| C-20'    | 12.80  | 12.79  | 12.77  | 12.76  | 12.68       |

$^a$ - $^d$: Corresponding assignments may be interchanged.

$Z$-methyl signals are underlined.
Compounds 5Z-L, 9Z-L, and 13Z-L are not only present in foods, having been reported to be produced through isomerization of lycopene in the stomach as a result of low pH after ingestion\textsuperscript{31}. Their bioavailabilities have been reported to be superior to that of AE-L because they are preferentially absorbed as mixed micelles in the intestine\textsuperscript{16, 32}.

4 Conclusion

In this study, we reported the \(^1\)O\(_2\) quenching activities of 5Z-l, 9Z-l, 13Z-l, and prolycopene, which are contained in common foods. We also proposed a simple method of distinguishing mono-Z-lycopenes using their Z-methyl \(^{13}\)C NMR chemical shifts. The \(^1\)O\(_2\) quenching activities and 3T3-L1 adipocyte differentiation activities of 5Z-L, 9Z-L, 13Z-L, and prolycopene reported in this study may provide hope that their inclusion in foods could help to prevent atherosclerosis and diabetes. Further in vivo studies of the effects of 5Z-L, 9Z-L, 13Z-L, and prolycopene on arteriosclerosis and diabetic models are in progress.

Supporting Information

This material is available free of charge via the Internet at http://dx.doi.org/jos.69.10.5650/jos.ess20163

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Biological Activities of Z-Lycopene Contained in Food

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