Isolation of Eriocitrin (Eriodictyol 7-rutinoside) from Lemon Fruit (Citrus limon BURM. f.) and Its Antioxidative Activity

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An antioxidant was isolated from the peel and juice of lemon fruit (Citrus limon BURM. f.). It was identified as eriocitrin (eriodictyol 7-rutinoside) of the flavanone glycoside by HPLC, 1H-NMR and 13C-NMR analyses. The purified eriocitrin was readily soluble in water, methanol, and ethanol. A water solution of 0.05% eriocitrin was weakly acidic (pH 4.2). Eriocitrin was found to be stable even at high temperature (121°C, 15 min) in acidic solution (pH 3.5). The distribution of eriocitrin in citrus fruits was found to be especially abundant in lemons and limes, however, it was scarcely found in other citrus fruits. In the case of lemon fruit, eriocitrin was primarily distributed in the peel (about 1,500 ppm) composed of the albedo (mesocarp), flavedo (epicarp), and pulp vesicles. It was also significantly present in the juice (about 200 ppm) but was not detected in the seed. Two varieties of lemon fruits, eureka and lisbon, almost had the same eriocitrin content. The antioxidative activity of eriocitrin in the linoic acid autoxidation system was equal to that of α-tocopherol, and it was enhanced when used together with citric acid. The eriocitrin had a synergistic effect on α-tocopherol.

Keywords: lemon fruit, Citrus limon, antioxidant, eriocitrin, flavanone glycoside

Lipid peroxidation is known as one of the major factors causing deterioration during the storage and processing of food. Lipid peroxidation is also speculated to be strongly associated with aging and carcinogenesis (Harman, 1982; Cutler, 1984; Yagi, 1987). Recently, it was reported that natural antioxidants contained in dietary plants may play an important role in the prevention of carcinogenesis and in extending the life span of animals and may offer effective protection from peroxidative damage in living systems (Osawa et al., 1990; Cutler, 1992; Jacob, 1995). Therefore, much attention has been focused on the importance of natural antioxidants, and several different types of antioxidants isolated from natural sources with high activity have been reported (Matsuzaki & Hara, 1985; Nishina et al., 1992; Ioku et al., 1992; Katsuzaki et al., 1993; Tsuda et al., 1994).

Lemon (Citrus limon BURM. f.) is one of the most popular citrus fruits in the world. Lemon juice is manufactured by squeezing the lemon fruits. At the same time, the peel and the seed are produced as a byproduct during the manufacturing process. The juice has been used in many kinds of foods, for example, soft drinks, alcoholic drinks, seasonings, and candies. The peel is partially used for foods such as jams, food materials such as essential oils, and citrus pectin, or is used for domestic animal food. The seed is partially used in materials such as essential oils, and citrus pectin, or is used for domestic animal food. The seed is also used in some domestic animal food.

Lemon has been known as a typical healthy food for a long time, and it has been reported that lemon juice has a desmutagenic effect (Achiwa et al., 1991) and an antimutagenic effect (Jain et al., 1987). Lemon fruits contain a number of nutrients such as citric acid, ascorbic acid, minerals, and flavonoids. Ascorbic acid, which is the so-called vitamin C, abundantly exists in the lemon juice. It has also been reported as an antioxidant with biofunctional activities in vivo (Jacob, 1995).

In this study, we paid much attention to the antioxidants except for ascorbic acid in the lemon fruit and to isolating the antioxidants from the peel and the juice of the lemon fruit. We expect that the new antioxidant in lemon fruit will provide protection from peroxidative damage in living systems in relation to aging and carcinogenesis. The antioxidative properties of the new antioxidant were also examined for use in foods.

Materials and Methods

Chemicals Linoleic acid and α-tocopherol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka). Eriodictyol was obtained from Funakoshi, Ltd. (Tokyo). Lemons (Citrus limon BURM. f.) and the other citrus fruits were purchased from a supermarket. The varieties of lemon, eureka and lisbon, were obtained from the Citrus Research & Education Center (Lake Alfred, Florida, USA). Some enzymes were also obtained: β-glucosidase of almond from Wako Pure Chemical Industries, Ltd., naringinase from Sankyo Pharmaceutical Company, Ltd. (Tokyo), hesperidinase from Tanabe Pharmaceutical Company, Ltd. (Tokyo), and pectinase from Amano Pharmaceutical Company, Ltd. (Nagoya).

Purification of the antioxidant from lemon fruit The antioxidant in lemon fruit was purified from the peel and juice of lemons. The peel (1.60 kg) obtained from 20 lemon fruits was chopped (5 mm×5 mm) in a homogenizer and extracted with hot water (6 l) for 2 h. The extract was filtered through a cloth to remove the peel and was concentrated in
Isolation of Eriocitrin from Lemon Fruit

The peel extract was then dissolved in water (300 ml). The solution was chromatographed on a Cosmosil 75C 18-OPN ODS column (Nacalai Tesque, Inc. (Kyoto), φ37×500 mm). The column was washed with 2 l of water and successively eluted with 20% methanol-water, 40% methanol-water, and methanol (2 l each). The 40% methanol-water portion was concentrated to about 5 ml in vacuo. Preparative high-performance liquid chromatography (HPLC) was carried out using a Shim-pack PREP ODS(L) column (Shimadzu Co., Ltd. (Kyoto), 50×250 mm) with a UV spectrophotometric detector (280 nm) and 40% methanol as the solvent at a flow rate of 80 ml/min.

Also, 100% lemon juice (7.25 l) from about 150 lemon fruits was centrifuged at 5,000 rpm to remove the pulp. The supernatant liquid of the juice were chromatographed on a Cosmosil 75C18-0PN ODS column as well as using the method for the lemon peel. The antioxidative sample (LE-A) was isolated from the peel and juice of the lemons.

Examination of the properties of LE-A

The physical and chemical properties of LE-A was examined. The form and the dry color, the pH of the solution containing 0.05% LE-A, the ultraviolet absorption, the coloration test with added FeCl₃, and the conversion of β-glucosidase were determined. The β-glucosidase was used as the purified reagent from almond. Naringinase, hesperidinase, and pectinase were also used as the enzymes having β-glucosidase activity. A final concentration of 0.5% for each enzyme was added to 100 mM sodium phosphate buffer solution (pH 5.0) containing 1 mg/ml eriocitrin. The solution was incubated at 37°C overnight and was subsequently analyzed by conversion of LE-A during the HPLC analysis.

Preparation and analysis of aglycone of LE-A

LE-A was treated with β-glucosidase to separate the sugar and the aglycone, and the aglycone of LE-A was purified by preparative HPLC. A final concentration of 1 mg/ml pectinase and 0.5% LE-A was added to 100 mM sodium phosphate buffer solution (pH 5.0). The solution was incubated at 37°C for 17 h, and the aglycone of LE-A was successively purified by preparative HPLC according to the method of LE-A purification. The aglycone of LE-A was submitted to HPLC, 1H-NMR, and 13C-NMR analyses for identification.

Antioxidative assay

Antioxidative activity was evaluated using the linoleic acid system (Osawa & Namiki, 1981). Each sample was added to a solution mixture of linoleic acid (0.13 ml) and 99.0% distilled ethanol (10 ml) with a 50 mM phosphate buffer (pH 7.0, 10 ml), and the volume was adjusted to 25 ml with distilled water. The solution was mixed in a conical flask and incubated at 40°C. At intervals during the incubation, the degree of oxidation was measured using the thiocyanate method (Mitsuda et al., 1966) by recording the absorbance at 500 nm after coloring with FeCl₃ and thiocyanate. α-Tocopherol (Toc.) was used as the positive standard.

Instrumental analysis

1H-NMR and 13C-NMR spectra were obtained using a JEOL JNM-EX-270 NMR instrument (270 MHz for 1H and 67.5 MHz for 13C) in d-CD3OD containing tetramethylsilane (TMS) as the internal standard. The identification of LE-A and the aglycone of LE-A obtained by hydrolysis using β-glucosidase was done using HPLC, 1H-NMR and 13C-NMR analyses.

Determination of eriocitrin in citrus fruits

Each citrus fruit was separated into the peel and juice by squeezing the fruit. The peel (8.0 g) was homogenized and extracted with 40 ml boiling hot water for 120 min. The juice was removed from the pulp by filtration. In order to determine the location of eriocitrin, the lemon fruit was separated into the peel, juice, and seed. The peel was further separated into the flavedo (epicarp), albedo (mesocarp), and pulp vesicles using a knife. Samples (8.0 g) of the subdivided peel and the seed were homogenized and extracted with 40 ml hot water for 120 min, and the pulp of the juice was removed by filtration. Eriocitrin in the extract of the peel and filtered juice were determined by HPLC analysis. HPLC (LC-10A, Shimadzu Co., Ltd.) was carried out using a Shim-pack CLC-ODS(M) (4.6×250 mm) with a UV detector (λ=280 nm). The solvent system contained 70% of a 5% acetic acid solution and 30% methanol. The flow rate was 1.0 ml/min.
Stability of eriocitrin by heat treatment at different pH

The buffer solution was prepared at pH 2 and pH 3.5 using a 100 mM sodium citrate-HCl buffer, and at pH 5.0 and pH 7.0 using a 100 mM sodium phosphate buffer. The final concentration of 0.1% eriocitrin was added to the different buffer solutions, and the solutions were heated at 80°C for 30 min or at 121°C for 15 min. The stability of eriocitrin by heat treatment was determined by the residual ratio of eriocitrin after heat treatment. The residual ratio of eriocitrin was determined from the residual peak of eriocitrin after heat treatment. The residual ratio of eriocitrin was obtained from 7.25 l juice from about 150 lemon fruits.

Results and Discussion

Isolation and identification of antioxidant in lemon fruit

We attempted the isolation of the antioxidant from the lemon peel and juice. Figure 1 shows the scheme for isolation of the antioxidant from the lemon fruit, and Fig. 2 shows the antioxidative activity from the purification process of the antioxidant from the lemon fruit.

For isolation from the peel, the homogenized peel was extracted with hot water. The antioxidant could also be extracted with methanol or ethanol (data not shown). The peel extract was fractionated by ODS column chromatography. The 40% methanol fraction exhibited strong antioxidative activity (Fig. 2), and preparative HPLC was then carried out. The antioxidative LE-A was purified (Fig. 1) with 1.47 g of LE-A being obtained from 1.60 kg peel of 20 lemon fruits.

For isolation from the juice, the pulps were excepted by centrifugation. The supernatant was fractionated by ODS column chromatography. The 40% methanol fraction exhibited strong antioxidative activity (Fig. 2) as did the peel extract. The antioxidative LE-A was purified from the juice (Fig. 1) with 0.1% eriocitrin was added to the different buffer solutions, and the solutions were heated at 80°C for 30 min or at 121°C for 15 min. The stability of eriocitrin by heat treatment was determined by the residual ratio of eriocitrin after heat treatment. The residual ratio of eriocitrin was determined from the residual peak of eriocitrin after heat treatment.

Identification of LE-A

LE-A was characterized by UV absorbance and the FeCl₃ test as shown in Table 1. The ultraviolet absorption showed a maximum at 210 nm, 280 nm, and 340 nm and the FeCl₃ test was positive. We postulated that LE-A was one of the flavonoid compounds because these data show the properties of a flavonoid compound. Enzyme treatment of LE-A shows conversion of LE-A (Table 1). The retention time of LE-A during HPLC analysis based on the agreement in retention times. The yield of LE-A from the peel was much greater than that from the juice.

As shown in Table 1, the purified LE-A was a pale yellow powder which was soluble in water, methanol, and ethanol. A water solution of 0.05% LE-A was acidic (pH 4.24) and had no taste. We confirmed that the taste of a water solution of 0.1% LE-A was slightly bitter, but it did not influence the taste of any food and drink. Eriocitrin seemed appropriate for use in food and beverages based on the properties of its water solubility and taste.

Identification of LE-A

LE-A was characterized by UV absorbance and the FeCl₃ test as shown in Table 1. The ultraviolet absorption showed a maximum at 210 nm, 280 nm, and 340 nm and the FeCl₃ test was positive. We postulated that LE-A was one of the flavonoid compounds because these data show the properties of a flavonoid compound. Enzyme treatment of LE-A shows conversion of LE-A (Table 1). The retention time of LE-A during HPLC analysis was changed by treatment with β-glucosidase. It shows that LE-A was hydrolyzed by treatment with β-glucosidase. We guessed that LE-A was a flavonoid glycoside having the aglycone of a flavonoid and the sugar.

Identification of LE-A and the aglycone of LE-A was
Isolation of Eriocitrin from Lemon Fruit

Table 2. 1H, 13C-NMR spectral data for aglycone of LE-A.

<table>
<thead>
<tr>
<th>Aglycone of LE-A</th>
<th>Eriodictyol</th>
<th>Aglycone of LE-A</th>
<th>Eriodictyol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak No.</td>
<td></td>
<td>Peak No.</td>
<td></td>
</tr>
<tr>
<td>H2</td>
<td>5.21 (13, 3)</td>
<td>5.28 (13, 3)</td>
<td>44.3</td>
</tr>
<tr>
<td>H3a</td>
<td>3.00 (17, 13)</td>
<td>3.06 (17, 13)</td>
<td>80.7</td>
</tr>
<tr>
<td>H3b</td>
<td>2.64 (17, 3)</td>
<td>2.70 (17, 3)</td>
<td>96.5</td>
</tr>
<tr>
<td>H6</td>
<td>5.85 (2)</td>
<td>5.90 (2)</td>
<td>97.3</td>
</tr>
<tr>
<td>H8</td>
<td>5.83 (2)</td>
<td>5.88 (2)</td>
<td>103.6</td>
</tr>
<tr>
<td>H2'</td>
<td>6.86</td>
<td>6.91</td>
<td>115.0</td>
</tr>
<tr>
<td>H5'</td>
<td>6.73 (1)</td>
<td>6.78 (1)</td>
<td>116.5</td>
</tr>
<tr>
<td>H6'</td>
<td>6.72 (1)</td>
<td>6.78 (1)</td>
<td>119.5</td>
</tr>
</tbody>
</table>

*a* Aglycone of LE-A was measured in CD30D with TMS as internal standard.

*b* The data had been reported by Kumamoto et al. (1984). Eriocitrin was measured in CD30D with TMS as internal standard.

Eriodictyol (5,7,3',4'-tetrahydroxyflavanone)

$C_{15}H_{12}O_6$, MW = 288.25

Eriocitrin (eriodictyol 7-rutinoside, Eriocitrin 7-rutinoside)

$C_{27}H_{32}O_{15}$, MW = 596.55

Rham = Rhamnose

Glc = Glucose

<table>
<thead>
<tr>
<th>Table 3. 13C-NMR spectral data of LE-A.</th>
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<tbody>
<tr>
<td>LE-A</td>
</tr>
<tr>
<td>Sugar</td>
</tr>
<tr>
<td>Aglycone</td>
</tr>
<tr>
<td>Sugar</td>
</tr>
<tr>
<td>Aglycone</td>
</tr>
<tr>
<td>Peak No.</td>
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<td>Peak No.</td>
</tr>
<tr>
<td>1</td>
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<td>14</td>
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<tr>
<td>15</td>
</tr>
</tbody>
</table>

*a* LE-A was measured in CD30D with TMS as internal standard.

*b* The data had been reported by Kumamoto et al. (1984). Eriocitrin was measured in CD30D with TMS as internal standard.

Stability of eriocitrin by heat treatment at different pH.

- temperature: 80°C for 30 min is used for the sterilization of yeast or mold, and that of 121°C for 15 min is used for the sterilization of heat stable bacteria.
- Eriocitrin was stable during heat treatment at 80°C for 30 min in acidic solution (pH 2.0, pH 3.5, and pH 5.0). Eriocitrin was found to be stable even at higher temperatures (121°C, 15 min) in a solution of pH 3.5, but its stability was weak in a neutral solution (pH 7.0). A part of the eriocitrin seemed to hydrolyze at 121°C after 15 min in a solution of pH 2.0. These results suggest that the use of eriocitrin seems to be suitable for acidic beverages and foods.

Attempted using HPLC, 1H-NMR, and 13C-NMR analyses. As shown in Table 2, the aglycone of LE-A agreed with eriodictyol (5,7,3',4'-tetrahydroxyflavanone) as a standard based on the 1H-NMR and 13C-NMR spectral data. This result shows that the aglycone of LE-A was identified as eriodictyol. Also, it was confirmed that the retention time of the aglycone of LE-A was compatible with that of eriodictyol during HPLC analysis. Therefore, LE-A appeared to be a eriodictyol glycoside. As shown in Table 3, the 13C-NMR spectra data for LE-A were compatible with those of eriocitrin (eriodictyol 7-rutinoside), which has already reported (Kumamoto et al., 1984). LE-A was identified as eriocitrin by 13C-NMR analysis. It has been reported that eriocitrin was isolated from lemon peel (Horowitz & Gentili, 1960; Vandercook & Stephenson, 1966). However, the antioxidative effect of eriocitrin has never been reported. We determined the antioxidative function of eriocitrin in lemon fruit.

Flavonoids that exhibit beneficial effects on capillary permeability and fragility were once known as vitamin P. The flavonoids in lemon fruit have been investigated with respect to many physiological functions (Middleton & Kandaswami, 1994). Many flavonoid glycosides have been reported to be abundant in citrus fruits such as hesperidin and naringin (Albach & Redman, 1969). Flavonoid glycosides in lemon peel have also been reported to have a hypotensive effect (Kumamoto et al., 1984). We expect that eriocitrin has various physiological functions which will be investigated in the future.

Stability of eriocitrin by heat treatment at different pH.

We examined the stability of eriocitrin toward sterilization because it is an important property for application to beverages and foods. Table 4 shows the stability of eriocitrin to heat treatment in different buffer solutions containing eriocitrin. The residual ratio of eriocitrin after each heat treatment was determined from the residual peak of eriocitrin after heat treatment by HPLC analysis. The stability of eriocitrin by heat treatment was determined by the residual ratio of eriocitrin after heat treatment. The residual ratio of eriocitrin was determined from the residual peak of eriocitrin after heat treatment by HPLC analysis.
Table 5. Distribution of eriocitrin in citrus fruits.

<table>
<thead>
<tr>
<th>Citrus fruits</th>
<th>Eriocitrin (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peel</td>
<td>Juice</td>
</tr>
<tr>
<td>Citrus limon (lemon)</td>
<td>1.540</td>
</tr>
<tr>
<td>C. aurantifolia (lime)</td>
<td>1.280</td>
</tr>
<tr>
<td>C. paradisi (grapefruit)</td>
<td>n.d.</td>
</tr>
<tr>
<td>C. sinensis (valencia orange)</td>
<td>n.d.</td>
</tr>
<tr>
<td>C. grandis (bantana)</td>
<td>n.d.</td>
</tr>
<tr>
<td>C. hassaku (hassaku)</td>
<td>n.d.</td>
</tr>
<tr>
<td>C. iyo (iyo)</td>
<td>trace&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. sphaeroarpa (kabosu)</td>
<td>n.d.</td>
</tr>
<tr>
<td>C. reticulata (ponkan)</td>
<td>n.d.</td>
</tr>
<tr>
<td>C. unshiu (unshiu)</td>
<td>trace</td>
</tr>
<tr>
<td>C. junos (yuzu)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fortunella japonica (kinkan)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 6. Distribution of eriocitrin in various locations in lemon fruit.

<table>
<thead>
<tr>
<th>Peel</th>
<th>Juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>flavedo (epicarp)</td>
<td>952</td>
</tr>
<tr>
<td>albedo (mesocarp)</td>
<td>908</td>
</tr>
<tr>
<td>pulp vesicles</td>
<td>1,170</td>
</tr>
<tr>
<td>Juice</td>
<td>174</td>
</tr>
<tr>
<td>Seed</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

<sup>a</sup> Not detected.

Distribution of eriocitrin in citrus fruits. The distribution of eriocitrin in citrus fruits was investigated. As shown in Table 5, the determination of eriocitrin from the peel and juice of citrus fruits was carried out using HPLC analysis. Eriocitrin was shown to also exist in limes as well as lemons, but very little was present in the other types of citrus fruits. Eriocitrin was found to be especially rich in lemon and lime fruits, and it was abundant in the peel obtained as a byproduct from juice factories. These results suggested that eriocitrin as an antioxidant can become available by the effective utilization of the peel.

As shown in Table 6, the content of eriocitrin in various parts of the lemon fruit was examined, and the difference in concentration in the varieties of eureka and lisbon was also examined. Eriocitrin abundantly existed at about 1,000 ppm in the flavedo (epicarp, yellow exterior of the peel), albedo (mesocarp, white interior of the peel), and pulp vesicles (in part of the enveloping flesh) in the lemon peel. The difference in the amount of eriocitrin in these locations in the peel was not significant. The juice contained about 200 ppm eriocitrin; however, it was not found in the seed. The concentration of ascorbic acid, which is known to be included in lemon juice, was about 450 ppm. Eriocitrin exists at about half the level of ascorbic acid in lemon juice. It exists in a considerable amount in lemon juice. The content of eriocitrin was similar in the eureka and lisbon varieties of lemon fruit. Little difference in content was found among the lemon varieties.

Multiplier effect on antioxidative activity of eriocitrin. Effective natural antioxidants such as α-tocopherol (Toc.) have been used in food industries, but it has been a problem because of its expense or water insolvibility. As shown in Fig. 3, the antioxidative activity of 10 µM eriocitrin in the linoleic acid system was similar to that of 10 µM α-tocopherol. The antioxidative activity for the addition of both 5 µM eriocitrin and 5 µM α-tocopherol was stronger than that of 10 µM eriocitrin or 10 µM α-tocopherol. We found that eriocitrin shows a synergistic effect with α-tocopherol. It was suggested that eriocitrin is an effective antioxidant for use in food to decrease the addition of α-tocopherol and for its easy use in beverages based on its water solubility. Citric acid, which is present at about 6% in lemon juice, is also commonly used as an antioxidant in foods due to its chelating effect on minerals (Fe²⁺, Cu²⁺). The antioxidative activity of citric acid in the linoleic acid system was found to be weak, but it enhanced the antioxidative activity of eriocitrin as a cooperative effect. It was suggested that an effective method for application of the antioxidative eriocitrin in food was to use it together with citric acid. We proposed that the combined use of eriocitrin and citric acid for food applications will be acceptable to consumers, because both can be obtained from natural lemons.

Ascorbic acid, which abundantly exists in lemon juice, is known as an antioxidant with biofunctional activity in vivo (Jacob, 1995). We expect that the antioxidative effect of lemon juice in living systems is also related to the antioxidative eriocitrin. We will attempt to investigate the antioxidative activity of eriocitrin in vivo, and we expect it to be useful for protection against peroxidative damage in living systems in relation to aging and carcinogenesis.

Fig. 3. Antioxidative activity of eriocitrin, citric acid, and α-tocopherol. Toc., α-tocopherol; C.A., citric acid; ERI, eriocitrin.
References


