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Changes in Esculeoside A Content in Different Regions of the Tomato Fruit during Maturation and Heat Processing

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ABSTRACT: We previously demonstrated that esculeogenin A, a new aglycone of the tomato sapogenol esculeoside A, inhibits both acyl coenzyme A:cholesterol acyl-transferase (ACAT)-1 and -2 and ameliorates the pathogenesis of atherosclerosis in apoE deficient mice. Although we believe that daily intake of esculeoside A from tomato products can play a beneficial role in preventing the pathogenesis of atherosclerosis, the compound is not being used for preventive medicine due to the lack of information on methods for quantitative analysis and the content and stability of the compound in tomato products. In the present study, we report the development of a high-performance liquid chromatography (HPLC) method using an instrument equipped with a refractive index (RI) detector for esculeoside A quantification. We used this method to measure the changes in esculeoside A content during maturation, its distribution in the fruit body, and its stability during the heating process. The contents of esculeoside A in cherry tomatoes and Momotaro tomatoes were 21- and 9-fold, respectively, higher than that of lycopene, which is the most well-known compound in tomatoes. Furthermore, the esculeoside A content in pericarp wall was higher than in the whole tomato fruit and increased in a time-dependent manner during maturation. Although the melting point of purified esculeoside A was 225 °C, the esculeoside A in crude tomato extract decreased in a temperature-dependent manner. Degradation due to the heating process was inhibited under a pH of 9. These results demonstrated that the esculeoside A content differs in the various types of tomatoes, during maturation, and during the heating process used for preservation.

KEYWORDS: Esculeoside A, Esculeogenin A, tomato, atherosclerosis, lycopene

INTRODUCTION

It is known that preventive medicine is the most important approach to preventing lifestyle-related diseases, such as atherosclerosis and type II diabetes, and improvements in the daily nutritional intake are therefore thought to help prevent the pathogenesis of these diseases. Because free cholesterol, which is incorporated into cells with modified low-density lipoprotein (LDL) through the scavenger receptors, is toxic to the cells, cholesterol is esterified to cholesterol ester (CE) by an acyl coenzyme A:cholesterol acyl-transferase (ACAT), which is an intracellular enzyme found in rough endoplasmic reticulum.[1] Cholesterol esterification changes macrophages to foam cells, which are characterized by intracellular accumulation of CE. ACAT-1 is found in Kuppfer cells of the liver, kidneys, and adrenal cortical cells, whereas ACAT-2 is mainly found in hepatocytes and intestinal mucosal cells.[2] These findings are consistent with the notion that ACAT-1 plays a critical role in foam cell formation, whereas ACAT-2 is responsible for the cholesterol absorption process in intestinal mucosal cells.[2]

We previously demonstrated that esculeogenin A, a new aglycon of esculeoside A[3] isolated from tomatoes by Nohara et al.,[4] significantly inhibited the accumulation of cholesterol ester in macrophages by inhibiting ACAT. The oral administration of esculeoside A to apoE deficient mice significantly reduced the levels of serum cholesterol and the areas of atherosclerotic lesions.[5] We believe that daily intake of esculeoside A from tomato products and supplements may play a beneficial role in preventing the pathogenesis of atherosclerosis. The tomato has attracted considerable attention since it was discovered to contain lycopene, a strong antioxidant.[6,7] Recent studies have reported the following constituents of tomato: a bitter component named TFI[8] was isolated from tomato seeds; a steroidal alkaloid glycoside, tomatine, and several spirosolane glycosides were obtained from the stems and leaves;[9] lactone,[10] pregnane,[11] and several spirosolane derivatives[12] from the roots of the tomato stock were reported. However, the steroidal alkaloid is thought to be absent in the ripe fruit.

For the discovery of esculeoside A, a simple procedure consisting of smashing the tomato by hand in water, followed by filtration, was carried out to produce the filtrate.[4] The filtrate was then subjected to polystyrene column chromatography. It was first eluted with water, followed by methanol. The methanolic eluate was subsequently subjected to reversed silica gel column chromatography to yield a major tomato saponin, esculeoside A, as colorless needles. It is possible that esculeoside A was not discovered for so long, despite extensive surveys of the composition analysis of tomatoes, because of its lack of UV absorbance. Therefore, the characteristics of esculeoside A in tomatoes were not well-known to date. In the present study, we report the development of a high-performance liquid

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chromatography (HPLC) method using an instrument equipped with a refractive index (RI) detector for esculeoside A quantification to measure the content changes of esculeoside A during maturation of tomato, the distribution of esculeoside A in the fruit bodies, and the stability of esculeoside A during the heating process.

MATERIALS AND METHODS

Chemicals. Methanol, hydrochloric acid, and phosphoric acid were purchased from Kanto Chemical (Tokyo, Japan). Lycopene, diethylenetriamine-*N*,*N*,*N*′,*N*′′,*N*′′-pentaacetic acid (DTPA), ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), α-tocopherol, aminoguanidine, and ascorbic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of the highest grade available from commercial sources. Pure tomato lines, including cherry (small, round-shaped), grape (small, oval-shaped), and Momotaro (large, pink color) were purchased from Japan Agricultural Cooperatives.

Determination of Esculeoside A by HPLC. Esculeoside A was purified from cherry tomatoes as described previously.[4] Esculeogenin A was then purified from the hydrolysate of esculeoside A as described previously.[4] Purified esculeoside A was dissolved in 4 mM HCl and used as an authentic standard. The amounts of esculeoside A and esculeogenin A were determined by an RI-201H RI detector (Shodex, Japan) with a Capcellpak C18 reverse phase column (4.6 × 250 mm, Shiseido, Japan) at a flow rate of 1 mL/min at 40 °C using a Shimadzu HPLC system equipped with LC-10AD pumps, a CTO-10A column oven, a DGU-12A degasser, a CBM-20A communication bus module, a SIL-20A autosampler, and LC solution (ver. 1.25) data processing software and using a mobile phase composed of 45% methanol/55% phosphoric acid buffer (pH 2.8).

Determination of Esculeoside A in Tomatoes. Because Esculeoside A was first identified from cherry and Momotaro tomatoes,[3,4] we chose these tomatoes to confirm that esculeoside A is detectable in tomato extracts using our HPLC system. Tomato samples (75 g each) were homogenized using an MX-X58 blender (Panasonic, Japan) in the presence of 200 or 150 mL water for cherry and Momotaro tomatoes, respectively, because of a difference of their densities, and then filtered. Amino acids and carbohydrates were removed with an HP-20 column (10 × 100 mm), and the adsorbed fraction was eluted with methanol (100 mL). The eluate was evaporated to 1 mL and applied to a Sep-Pak C18 solid-phase extraction cartridge (Nihon Waters, Tokyo, Japan). The methanol phase was evaporated to dryness with a centrifugal evaporator and redissolved in 1 mL of 50% methanol. The samples were diluted 10-fold, and then 20 μL of each sample was analyzed by an HPLC system as described above. To measure the localization of esculeoside A, cherry tomatoes and Momotaro tomatoes were disassembled into pericarp wall, seeds, and columella. Whole tomatoes (75 g), seeds (75 g), pericarp wall (75 g), and columella (25 g) were homogenized with 500 mL of water to increase the extraction efficiency. The samples were applied to HP-20 and Sep-Pak C18 solid-phase extraction cartridges and then were analyzed using the HPLC system described above.

Changes in Esculeoside A Content during Maturation of the Fruit Body. Cherry tomatoes and grape tomatoes were harvested on days 15 (immature), 29 (intermediate maturity), and 33 (mature) after flower abscission and stored at −30 °C until analysis. The tomatoes were defrosted, and each group (75 g) was homogenized with water (500 mL) and filtered. The other extraction processes and the HPLC analysis were the same as described above.

Determination of the Thermal Stability of Esculeoside A in Homogenized Tomatoes. Cherry tomatoes (225 g) were homogenized in 1500 mL of water, filtered, and then heated at 50−100 °C for 30 min or at 121 °C for 20 min, followed by determination of the esculeoside A content by HPLC, as described above. Furthermore, to determine the mechanism in which the esculeoside A content in homogenized tomato decreases by heating, the filtrate of the homogenized tomatoes (150 g of cherry tomato with 1000 mL of water) was divided into 10 portions and then boiled for 30 min in the presence of several compounds, such as 100 mM aminoguanidine, an aldehyde-trapping reagent, metal chelators such as 2 mM DTPA and 2 mM EDTA, 100 mM citric acid, and antioxidants such as 10 μM α-tocopherol, 10 mM ascorbic acid, and sodium hydroxide to adjust the pH. To measure the effect of pH adjustment on the esculeoside A content in homogenized tomatoes, the pH of homogenized tomatoes was adjusted from 4 to 11 by sodium hydroxide, followed by incubation at 100 °C for 30 min. The esculeoside A content was measured by HPLC as described above.

Comparison of the Extraction Efficiency of Esculeoside A from Tomatoes. Before the esculeoside A content was compared with that of lycopene, the conventional extraction method for esculeoside A from tomatoes was re-evaluated. Cherry tomatoes (100 g) were homogenized in a blender and divided into two portions. One portion (50 g) was mixed with 350 g of water and vigorously shaken for 10 min, and the filtrate was applied to an HP-20 column as described above. The adsorbed fraction on the HP-20 column was evaporated in vacuo and redissolved in 50% methanol (750 μL), followed by determination by HPLC. The other homogenized tomato portion (50 g) was mixed with 200 g of methanol to yield 80% methanol (w/w) and was vigorously shaken for 10 min, followed by centrifugation at 5000 rpm for 10 min. The supernatant was filtered, evaporated to dryness, and redissolved in 70% methanol (2 mL). The samples were then applied to HP-20 columns. The adsorbed fraction on the HP-20 column was analyzed by HPLC in the same manner as that used for the conventional extraction method described above.

Determination of Lycopene Content in Tomatoes. Lycopene was analyzed according to the method reported by Periago et al. with minor modifications.[13] Briefly, hexane (50 mL) was added to homogenized cherry tomatoes (10 g) and vigorously shaken for 10 min. The supernatant was collected after centrifugation at 1200 rpm for 5 min. Extraction using hexane was repeated again, and the hexane fraction was evaporated to dryness and redissolved in 1 mL of tetrahydrofuran. The amount of lycopene was determined by the HPLC system equipped with a UV−vis detector (SPD-10AV; Shimadzu) with a reverse phase column (Capcellpak C18, 4.6 × 250 mm, Shiseido, Japan) at a flow rate of 1.2 mL/min at 45 °C, with a degasser (DG-980-50, Jasco, Japan), ternary gradient unit (LG-980-02, Jasco), intelligent HPLC pump (PU-980, Jasco), and column oven (860-CO, Jasco). The mobile phase consisted of 100% acetonitrile, and the sample (20 μL) was injected into the HPLC system, and the effluent was monitored by absorbance at 470 nm.

Statistical Analysis. All experimental data are expressed as the mean ± SD. Differences between groups were examined for statistical significance using Student's *t* test. A P value of <0.05 denoted the presence of a statistically significant difference.

RESULTS AND DISCUSSION

Detection of Esculeoside A in Tomatoes by HPLC. Purified esculeoside A (Figure 1, inset) was applied to the HPLC, and the effluent was monitored with the RI detector as described under Materials and Methods. As shown in Figure 1, esculeoside A was eluted at the retention time of ∼13.2 min. Furthermore, purified esculeoside A was quantified linearly up to 31.5 nmol as standard (Figure 1, inset). We next detected esculeoside A in the fruit body of cherry tomatoes, a round-shaped small tomato, as shown in Figure 2, by HPLC. Esculeoside A, which was extracted from tomatoes as described under Materials and Methods, was

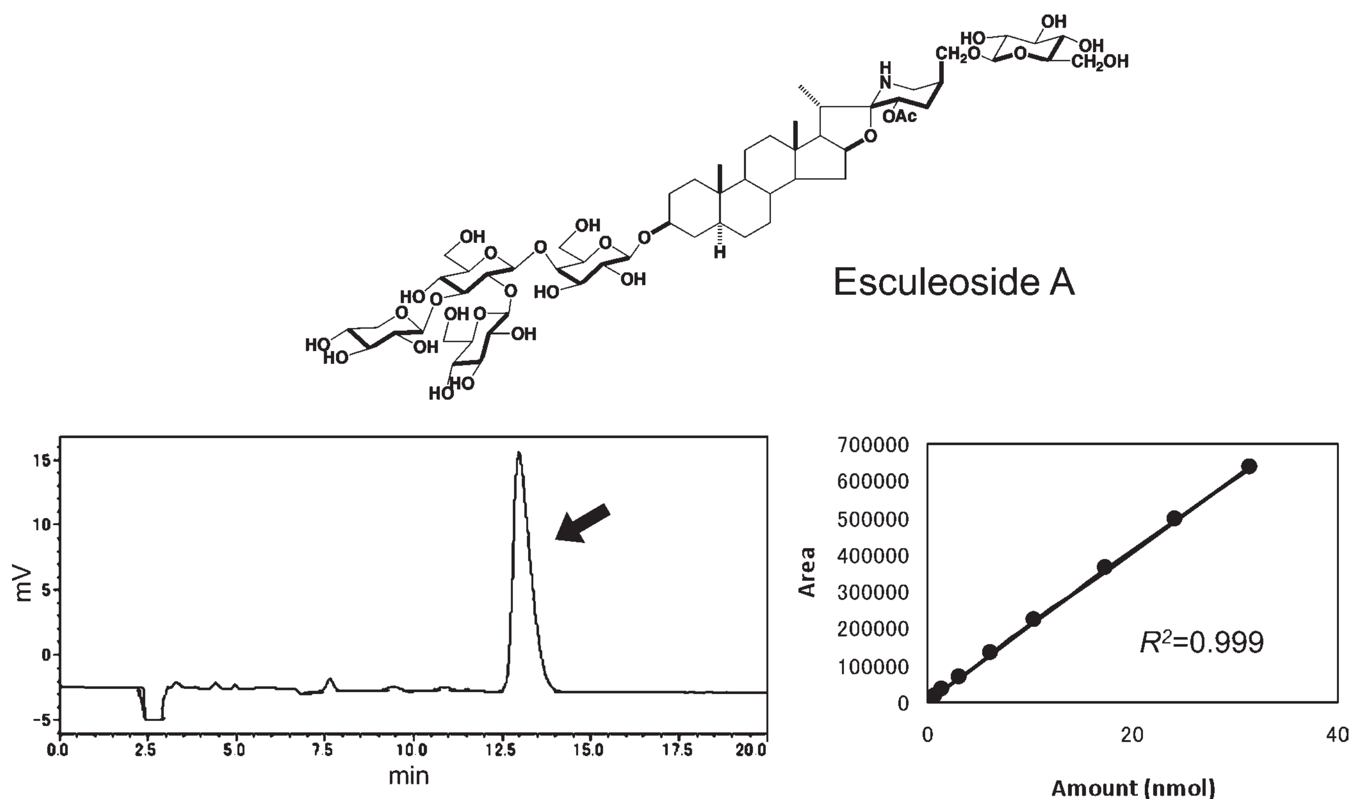

Figure 1. Detection of purified esculeoside A by HPLC. Purified esculeoside A (10.5 nmol) was analyzed by HPLC as described under Materials and Methods, and a standard curve of esculeoside A was obtained (inset).

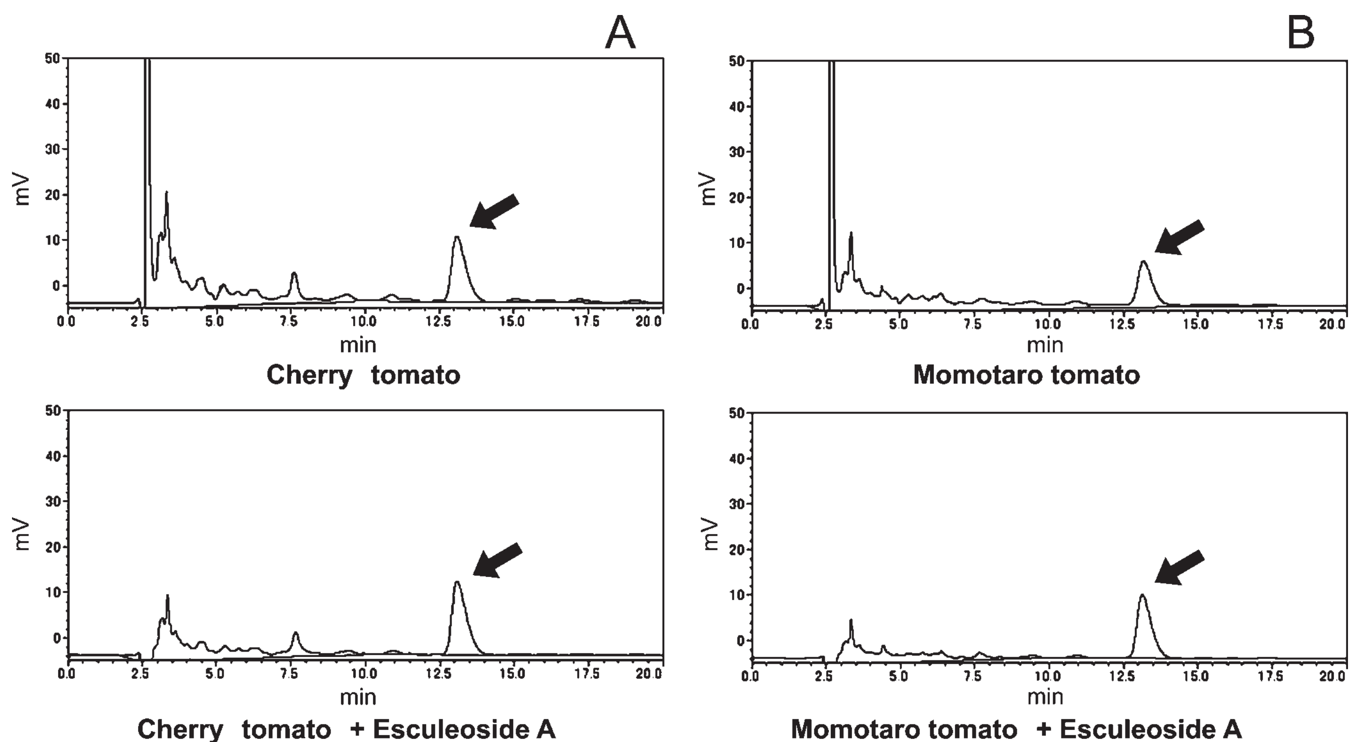

Figure 2. Detection of esculeoside A in tomatoes by HPLC. Esculeoside A was extracted from 75 g of cherry tomatoes (A) or Momotaro tomatoes (B) and analyzed by HPLC as described under Materials and Methods. The same samples were mixed with an equivalent volume of purified esculeoside A (1575 nmmol/mL) and injected again to confirm the retention time of esculeoside A in tomato samples.

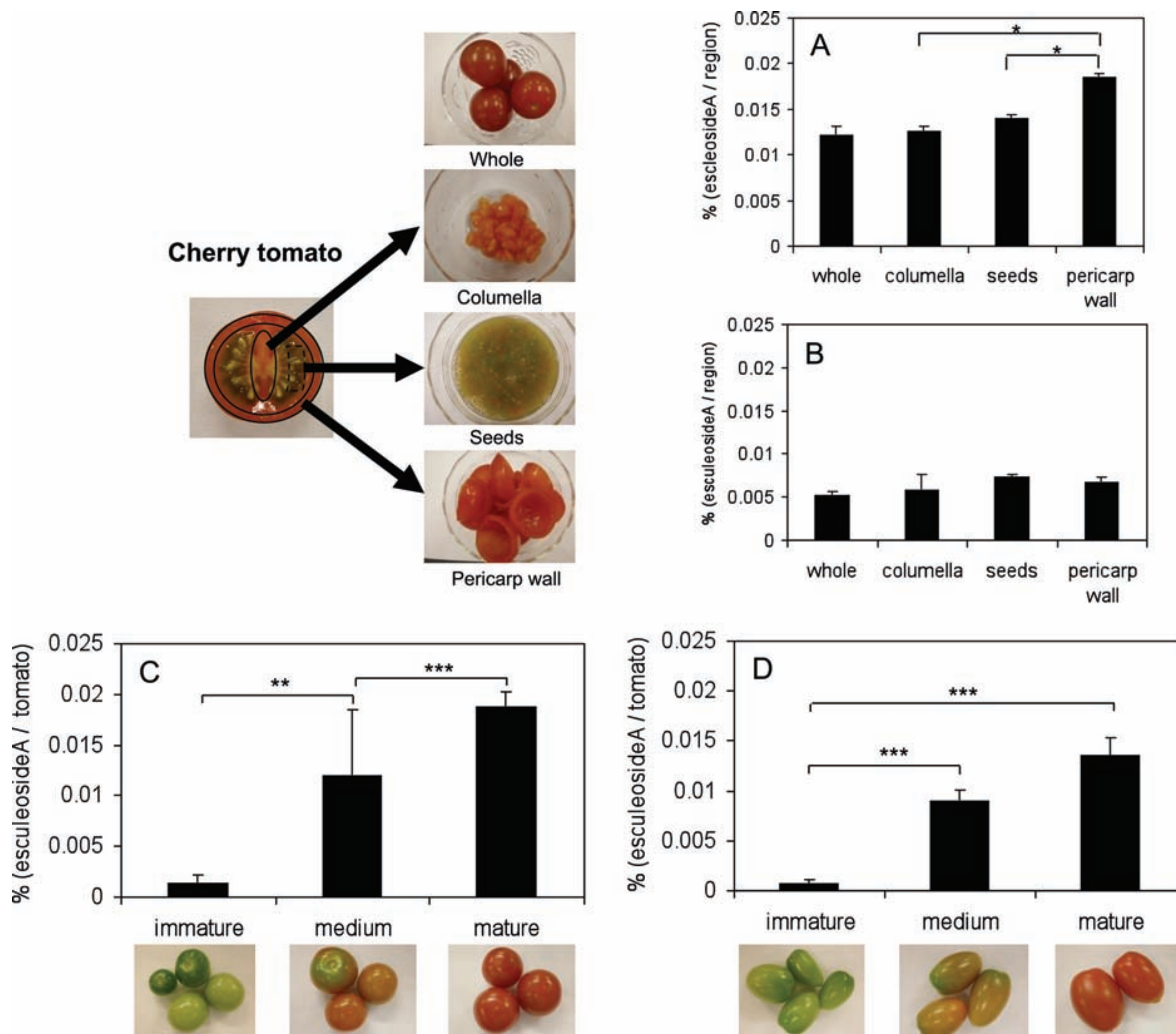

Figure 3. Distribution and changes in esculeoside A content in tomatoes. Cherry tomatoes (A) and Momotaro tomatoes (B) were disassembled into pericarp walls, seeds, and columellas. The esculeoside A content in each section was measured by HPLC. Cherry tomatoes (C) and grape tomatoes (D) were harvested on days 15 (immature), 29 (intermediate maturity), and 33 (matured) after flower abscission and stored at −30 °C until analysis. The tomatoes were defrosted, and the esculeoside A content was measured by HPLC. The data are presented as the mean ± SD (n = 3). ∗, $P < 0.0001$, versus pericarp wall. ∗∗, $P < 0.05$, and ∗∗∗, $P < 0.001$, versus immature.

observed in cherry tomatoes, and its peak area was increased when the sample was mixed with an equivalent volume of purified esculeoside A solution (2 mg/mL) (Figure 2A). The same tendency was observed for Momotaro tomatoes. Thus, esculeoside A was detected at ∼10 min, and its peak area was increased in the presence of purified esculeoside A (Figure 2B), thus indicating the specificity of the peak. However, the peak area of esculeoside A in Momotaro tomatoes was lower than that of cherry tomatoes (Figure 2). Although the retention time of esculeoside A changes by ∼0.2 min as a result of differences in the density of the samples, our methods allowed us to detect esculeoside A as a major peak in the tomato extracts (Figure 2).

Distribution of Esculeoside A in Tomatoes. To clarify the distribution of esculeoside A in tomatoes, the fruit bodies of cherry tomatoes and Momotaro tomatoes were divided into three portions, the pericarp wall, the seeds, and the columella, and the esculeoside A content was measured by HPLC. As shown in Figure 3A, the esculeoside A content in the pericarp wall was higher than in the other areas in cherry tomatoes, whereas the esculeoside A content was almost the same among the three regions in the Momotaro tomatoes (Figure 3B).

Changes in Esculeoside A Content during Maturation of the Fruit Body. Because the content of esculeoside A in cherry tomatoes was higher than that of Momotaro tomatoes, we next measured the changes in the esculeoside A content in small tomatoes during the maturation of the fruit body. As shown in Figure 3C, the esculeoside A content in cherry tomatoes (round-shaped) increased during maturation. The same tendency was observed in grape tomatoes (oval-shaped) (Figure 3D), although the esculeoside A content in grape tomatoes on day 33 was lower than that of cherry tomatoes (Figure 3C,D).

Thermal Stability of Esculeoside A in Homogenized Tomatoes. Although purified esculeoside A is stable until 225 °C,[4] the level of esculeoside A in commercial beverages was lower than the limit of detection.[14] To clarify this discrepancy, we next measured the stability of esculeoside A in homogenized fruit bodies during the heat sterilization process. Although the esculeoside A content was unchanged when a homogenized tomato

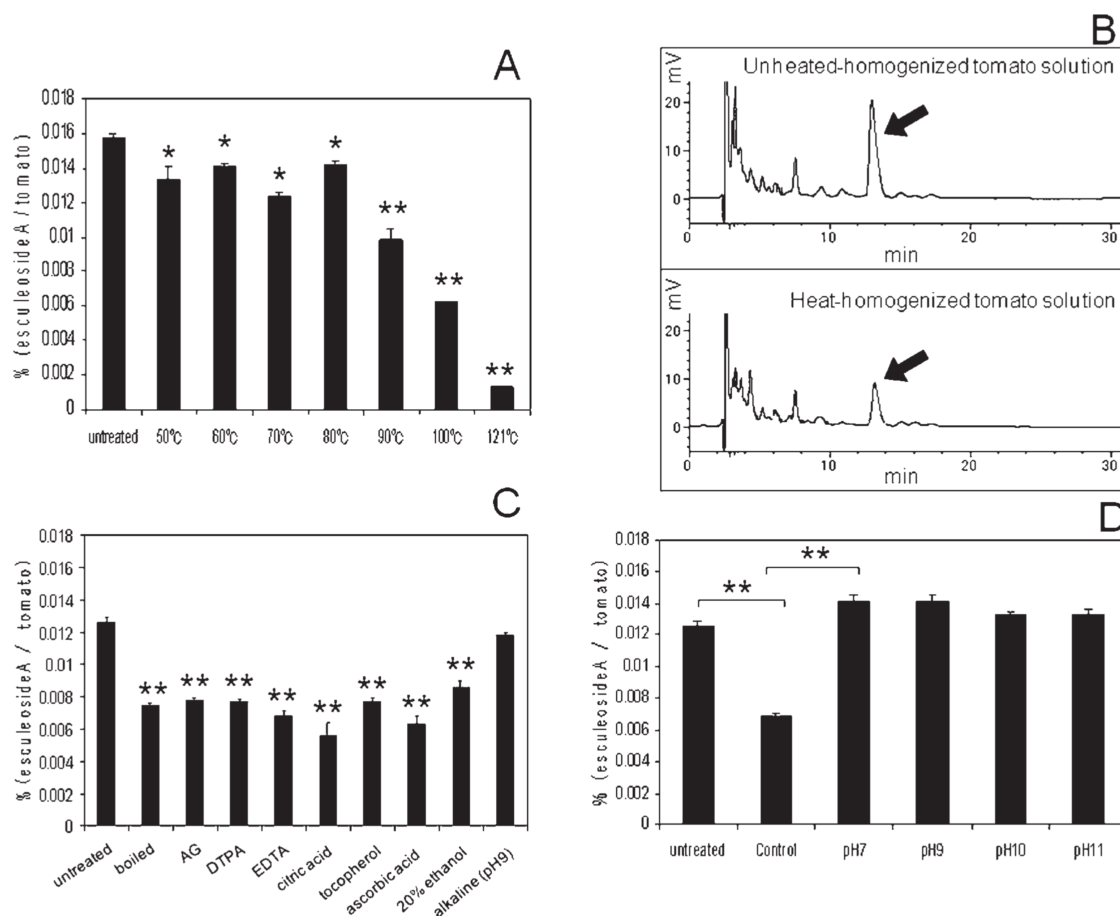

Figure 4. Thermal stability of esculeoside A in homogenized tomatoes. (A) The homogenized cherry tomato solution was heated at 50−100 °C for 30 min or at 121 °C for 20 min, followed by determination of the esculeoside A content by HPLC as described above. (B) Chromatograms of unheated and heated (at 100 °C for 30 min) tomato solutions. (C) The homogenized cherry tomato solution was heated at 100 °C for 30 min in the presence of 100 mM aminoguanidine (AG), 2 mM DTPA, 2 mM EDTA, 100 mM citric acid, 10 μM α-tocopherol, 10 mM ascorbic acid, and sodium hydroxide to adjust the pH. The esculeoside A content was measured by HPLC as described above. (D) The homogenized cherry tomato solution was heated at 100 °C for 30 min after changing the pH from 7 to 11, and then the esculeoside A contents were measured by HPLC. The data are presented as the mean ± SD (n = 3). ∗, $P < 0.01$, and ∗∗, $P < 0.001$, versus untreated samples.

solution was stored at 4 °C for 4 weeks (mean ± SD = fresh sample, 0.012 ± 0.0001; 4 week sample, 0.011 ± 0.0001), it decreased with increasing temperature when homogenized cherry tomato solution, prepared as described under Materials and Methods, was incubated for 30 min at the indicated temperatures (Figure 4A). The esculeoside A content in homogenized tomatoes was decreased by ∼75% after autoclaving at 121 °C for 20 min (Figure 4A). A chromatogram of the heated−homogenized tomato solution is shown in Figure 4B. A new peak did not appear between the retention time of esculeoside A at ∼13.2 min and that of esculeogenin A at ∼24 min after heating of the samples, indicating that not only the sugar moiety but also the steroidal alkaloid moiety of esculeoside A could be degraded by heating under acidic conditions.

To explore possible conditions that could prevent the decrease in esculeoside A content, we added several compounds to the homogenized tomatoes before incubation at 100 °C for 30 min, followed by measurement of esculeoside A content by HPLC. The aldehyde-trapping reagent, metal chelators, and antioxidants did not show any inhibitory effects on the heat-induced decrease in esculeoside A content in homogenized tomatoes (Figure 4C).

Table 1. Comparison of the Extraction Efficiency of Esculeoside A from Tomatoes

	% (esculeoside A/tomato)	SD
water extraction	0.011	0.000001
methanol extraction	0.024[a]	0.0001

[a] Cherry tomatoes (100 g) were homogenized, and the extraction efficiency between the conventional method by water extraction and methanol extraction was compared. Half of the homogenized tomato solution (50 g) was mixed with water and vigorously shaken for 10 min, followed by measurement of the esculeoside A content by HPLC as described under Materials and Methods. The other half of the homogenized tomato solution (50 g) was mixed with methanol and vigorously shaken for 10 min, followed by determination of esculeoside A content by HPLC using the same method described above. The data are presented as the mean ± SD (n = 3). *, $P < 0.0001$ versus water extraction.

Because the pH of homogenized cherry tomatoes was around 4, we also measured the effect of pH adjustment on the esculeoside A content in homogenized tomatoes. As shown in Figure 4C, heat-induced reduction of esculeoside A was inhibited at pH 9, indicating that alkaline conditions ameliorated the heat-induced

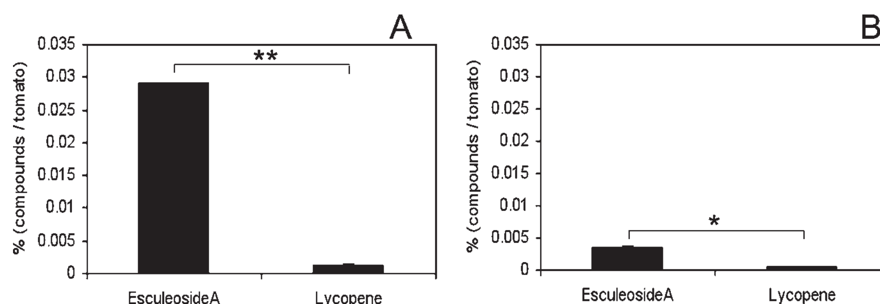

Figure 5. Comparison of the amounts of esculeoside A and lycopene present in tomatoes. Lycopene was extracted from tomatoes by hexane, and the amount of lycopene in cherry tomatoes (A) and Momotaro tomatoes (B) was determined by the HPLC system as described under Materials and Methods. The data are presented as the mean ± SD (n = 3). ∗, $P < 0.0001$ versus lycopene.

reduction of esculeoside A. To confirm this observation, the pH of homogenized tomatoes was adjusted from 4 to 11, followed by incubation at 100 °C for 30 min. As shown in Figure 4D, pH 7−11 inhibited the decrease in esculeoside A content by heating. Purified esculeoside A in water was stable even after incubation at 100 °C for 30 min. This result is consistent with the previous study by Fujiwara et al.[4] However, purified esculeoside A decreased by 55% when the esculeoside A solution (525 μM) was incubated at 100 °C for 30 min at pH 4. Taken together, our results demonstrated that esculeoside A is unstable when heated under acidic conditions.

Comparison of the Extraction Efficiency of Esculeoside A from Tomatoes. To compare the amount of esculeoside A and lycopene present in cherry tomatoes, we first compared the extraction efficiency of esculeoside A from tomatoes between the reported conventional method[3,4] and a new methanol extraction method. We employed 80% methanol to extract esculeoside A from tomato fruits because the extraction efficiency was the highest at this concentration (among concentrations 0−100%; data not shown) of methanol. As described under Materials and Methods, homogenized cherry tomatoes were extracted with water or 80% methanol (w/w), followed by determination of the esculeoside A content by HPLC. As shown in Table 1, the esculeoside A content determined following methanol extraction was significantly higher than that determined following water extraction, demonstrating that esculeoside A is more effectively extracted by methanol.

Comparison of the Amount of Esculeoside A and Lycopene Present in Tomatoes. As shown in Figure 5A, the esculeoside A content in cherry tomatoes was >21-fold higher than that of lycopene. The same tendency was observed in Momotaro tomatoes, with the esculeoside A content in Momotaro tomatoes being >9-fold higher than lycopene (Figure 5B). The esculeoside A content in Momotaro tomatoes was lower than in cherry tomatoes.

Although the structure of esculeoside A was reported in 2003,[3] little is known about the stability of esculeoside A in solution because there was not a conventional quantification system for this compound. Therefore, esculeoside A has not yet been used in functional foods or preventive medicines for lifestyle-related diseases despite its antiatherosclerotic activity via inhibition of ACAT.[5] Because the improvement of daily nutritional intake is thought to prevent the pathogenesis of lifestyle-related diseases such as atherosclerosis and type II diabetes, we believe that daily intake of esculeoside A from tomato products and supplements could play a beneficial role in preventing the pathogenesis of atherosclerosis. Our study provides the first evidence that purified esculeoside A in water at pH 7−11 is stable even during sterilization by heating, whereas it is unstable under acidic conditions. This information will be beneficial for developing new functional foods and preventive medicines.

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