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Report

Adenosine Acts as an Active Antiplatelet Constituent in Strawberries (*Fragaria* × *ananassa*)

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Many foods have been reported to be effective against thrombosis, but most of them may be difficult to ingest in effective doses to prevent the disease. In this study, we focused on strawberries as one of the foods reported to have antithrombotic activities because they are highly palatable to many people and have several intake methods (e.g., raw fruits, juices, jams, etc.). In this study, strawberry 'Minomusume' was selected as a material to explore antiplatelet active compounds. The activity-guided fractionation of the strawberry extract resulted in the active compound being adenosine. Furthermore, we investigated the correlation between adenosine content and the antiplatelet activity of strawberries. As a result, as for the cultivars whose adenosine was detected, significant positive correlation was observed.

Key words strawberry, *Fragaria* × *ananassa*, Minomusume, antiplatelet, adenosine, thrombosis

INTRODUCTION

According to the vital statistics in 2019 reported by the Ministry of Health, Labour and Welfare of Japan, the death ratio from thrombotic diseases, including heart and cerebrovascular diseases, was about 22.7%. This ratio is nearly up to that of malignant neoplasms (27.3%) and is increasing, especially if heart diseases are considered.[1] These diseases are recognized worldwide as severe causes of death. However, recognizing thrombosis as a common cause of these cardiovascular diseases is insufficient. Medication is a direct method to cure thrombosis, but prevention must be the best way to reduce the number of people who suffer from it. Lifestyle changes should also be attempted to prevent these life-threatening diseases, and improvements in daily diets are considered essential. So, many foods have been reported to be effective against thrombosis, but most of them may be difficult to ingest in effective doses to prevent the disease. Some vegetables, such as spinach (*Spinacia oleracea*),[2] parsley (*Petroselinum crispum*),[2] shiso (perilla) (*Perilla frutescens* var. *crispa*),[3] garlic (*Allium sativum*),[4] and turmeric (*Curcuma longa*),[5] are known to possess robust antithrombotic effects. All of these were obtained by *in vitro* studies. What is essential in investigating the antithrombotic activity of foods is whether their activity can be observed at an amount that can be ingested in a daily diet.

Furthermore, some foods have low palatability due to their unique tastes and odors. Such foods are not considered appropriate for preventing or treating diseases by dietary habits. For instance, onions and garlic are said to affect various diseases and have been reported to have antithrombotic activities.[4,6] Still, they are not highly palatable due to the odor peculiar to sulfides. In addition, ingesting such foods is often doubtful because of the lack of information regarding the daily consumption amounts that need to be digested to obtain their beneficial effects. On the other hand, among various vegetables and fruits with *in vitro* antithrombotic activity,[7] it has been reported that strawberries have an antithrombotic effect.[8,9] However, they did not speculate on compounds which contribute to the activity.

Strawberries are highly palatable to many people and are characterized by various intake methods. This fruit may be consumed raw, as juice, jam, and other heated products. Therefore, strawberry is considered a food suitable for disease prevention by improving diet. Accordingly, we focused on the antithrombotic activity of strawberries in this study. Before researching antithrombotic compounds, four cultivars commonly planted and distributed in Japan were evaluated for platelet aggregation inhibitory activity using ADP and collagen as stimulants, and fruits of 'Minomusume' cultivar was selected as a material for exploring antiplatelet active compounds in strawberry. *Fragaria* × *ananassa* Duch. 'Minomusume' (Rosaceae) was developed in a forced culture at the Gifu Prefectural Agricultural

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Technology Center. There is only one report on the constituents in 'Minomusume' strawberry fruits, but several anti-allergic compounds were reported on this cultivar in 2010.[10] We discuss that adenosine was the platelet aggregation-inhibiting compound contained in 'Minomusume' strawberries. We also observed a correlation between the amount of adenosine in strawberries and platelet aggregation-inhibiting activity.

MATERIALS AND METHODS

General ^{1}H- and ^{13}C-NMR spectra were recorded in methanol-d_4 on a JEOL A-600 spectrometer (JEOL, Tokyo, Japan) and a Bruker AvanceIII HD600 spectrometer (Bruker, MA, USA) equipped with a Prodigy liquid nitrogen cryoprobe. Chemical shifts for ^{1}H- and ^{13}C-NMR spectra in D_2O were given in ppm (δ) relative to the acetone signal (δ_H 2.225)[11] and dioxane signal (δ_C 67.15), respectively, as internal standards. Coupling constants (*J*) were expressed in Hz. Column chromatographic separations were conducted on silica gel (BW-820MH, Fuji Silysia, Aichi, Japan) and ODS gel (Develosil Lop ODS, Nomura Chemical CO., Ltd., Aichi, Japan). Analytical TLC was performed on precoated silica gel 60 F254 plates (Merck, Darmstadt, Germany). Spots were visualized under UV light at 254 nm and by spraying with anisaldehyde-sulfuric acid reagent, followed by heating at 200°C for 1 min. Semipreparative HPLC separations were performed on a chromatograph equipped with a multiwavelength detector (JASCO, Tokyo, Japan) using Develosil ODS HG-5 and Develosil C30 UG-5 columns (both φ 20 × 250 mm, Nomura Chemical CO., Ltd.) and Sunniest RP-AQUA (φ 20 × 250 mm, ChromaNik Technologies, Osaka, Japan). Analytical HPLC for quantification of adenosine was conducted using a Develosil C30 UG-5 column (φ 4.6 × 250 mm, Nomura Chemical CO., Ltd.), and H_2O:MeOH:CH_3CN = 171:9:10 as a mobile phase. The extraction of organic compounds from strawberry juice was conducted on an ion exchange resin, Amberlite XAD-2 (Organo Corp., Tokyo, Japan). Adenosine, inosine, and adenosine deaminase from the bovine spleen were the products of Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were analytical-grade products of TCI (Tokyo, Japan) and Kanto Chemical Company (Tokyo, Japan). An aggregometer was used to measure platelet aggregation, and this analysis was conducted on an MCM Hematracer 313M (MC Medical, Inc., Tokyo, Japan).

Strawberries The 'Minomusume' strawberry fruits were harvested from the Gifu Prefectural Agricultural Technology Center and were immediately frozen at –25°C.

Extraction and Fractionation After thawing 5.1 kg of frozen 'Minomusume' fruits, they were squeezed, and the obtained juice was centrifuged (7000 rpm, 4°C, 10 min). Amberlite XAD-2 (500 g) was added to the resulting supernatant, and the mixture was filtered to obtain a non-absorbed fraction (FA-I-1). The resin was eluted with 2 L of MeOH and acetone. The corresponding eluates were evaporated to obtain a MeOH-soluble fraction (FA-I-2) (30.2 g) and an acetone-soluble fraction (FA-I-3) (3.0 g).

The MeOH fraction (2.9 g) was fractionated using an ODS column chromatography with mixed solvents of H_2O-MeOH (9:1 and 1:1) and MeOH to obtain four fractions: FA-II-1 (585 mg), FA-II-2 (1.55 g), FA-II-3 (476 mg), and FA-II-4 (35 mg). FA-II-2 (1.48 g) was further separated by using the ODS column again with mixed solvents of H_2O-MeOH (9:1 and 1:1) and MeOH to obtain two fractions, FA-III-1 (220 mg) and FA-III-2 (1.04 g).

FA-III-2 (952 mg) was fractionated using HPLC using Develosil ODS-HG-5 with a mixture of H_2O:CH_3CN by the ratio of 9:1–8:2–0:10 at a flow rate of 3 mL/min, and four fractions of FA-IV-1 (312 mg), FA-IV-2 (203 mg), FA-IV-3 (391 mg) and FA-IV-4 (4 mg) were obtained. FA-IV-1 (192 mg) was separated into three fractions of FA-V-1 (75 mg), FA-V-2 (32 mg), and FA-V-3 (82 mg) using HPLC with Develosil C30-UG-5 using H_2O:CH_3CN = 9:1 as a mobile phase at a flow rate of 3 mL/min. FA-V-2 (26 mg) was purified using HPLC with Sunniest RP-AQUA using H_2O:CH_3CN = 9:1–85:15 and methanol as a mobile phase at a flow rate of 3 mL/min. Adenosine was obtained as FA-VI-3 (1.9 mg).

Adenosine: ^{1}H-NMR (600 MHz in D_2O) δ 8.33 (1H, s; H-8), 8.25 (1H, s; H-2), 6.07 (1H, d, J = 6.2 Hz; H-1′), 4.80 (1H, dd, *J* = 6.2, 4.9 Hz; H-2′), 4.43 (1H, dd, *J* = 4.9, 3.6 Hz; H-3′), 4.29 (1H, dt, *J* = 2.7, 3.6 Hz; H-4′), 3.91 (1H, dd, *J* = 12.8, 2.7 Hz; H-5′), 3.83 (1H, dd, *J* = 12.8, 3.6 Hz; H-5′); ^{13}C-NMR (150 MHz in D_2O) δ 156.4 (C-6), 153.3 (C-2), 149.3 (C-4), 141.3 (C-8), 119.9 (C-5), 88.9 (C-1′), 86.4 (C-4′), 74.2 (C-2′), 71.2 (C-3′), 62.1 (C-5′).

Platelet Aggregation Inhibitory Activity The platelet aggregation inhibitory activity was measured using a light transmission method[12] with a platelet-rich plasma (PRP) isolated from human peripheral blood. In this experiment, ADP and collagen were used as platelet-activating substances, and changes in the absorbance of PRP associated with platelet aggregation were measured over time using an aggregometer. A sample solution (5 μL) adjusted to 10 mg/mL was added to PRP (200 μL) and incubated at 37°C for 3 min. A total of 5 μL of ADP (100 μM) or collagen (20 μg/mL) was added to it. The change in absorbance was measured using an aggregometer to calculate the platelet aggregation rate (%). The platelet aggregation inhibition rate (%) was obtained using the following formula:

$$\text{Platelet aggregation inhibition rate (\%)} = \left(1 - \frac{\text{aggregation rate}_{\text{SAMPLE}}}{\text{aggregation rate}_{\text{CONT}}}\right) \times 100$$

The platelet aggregation inhibition rate of each sample was calculated by setting the platelet aggregation rate of the control group to 100%. A solvent for preparing a sample solution was used for the control.

When a supernatant prepared from the strawberry freeze-dried powder shown below was tested for antiplatelet activity, the supernatant (10 μL) was used instead of the sample solution mentioned above.

Preparation of Strawberry Samples for Screening of Antiplatelet Activity Strawberry fruits cultivated and harvested at the NARO Kyushu Okinawa Agricultural Research Center. The fresh fruits were crushed by a blender, and lyophilized. The resulted powder was suspended to a brine at a concentration of 100 mg/mL, and stirred for 12 h at 4°C. The suspension was centrifuged at 15000 rpm (4°C for 10 min), and the resulted supernatant was obtained. The supernatant was centrifuged again under the same conditions, and 10 μL of the supernatant was used for a sample of the antiplatelet test.

Quantification Analysis of Adenosine using HPLC The freeze-dried powder prepared for the screening of antiplatelet activity was used to quantify adenosine content. The powder (1 g) was suspended in distilled water (50 mL). The suspen-

sion was sonicated for 3 min and centrifuged (4°C, 3000 rpm, 25 min). Amberlite XAD-2 resin was added to the supernatant. The mixture was stirred for 20 min and filtered. The resin was eluted successively with methanol and acetone. The methanol eluate was evaporated to prepare an aqueous solution at 10 mg/mL.

The solution was analyzed using an HPLC equipped Develosil C30-UG-5 column, and adenosine content was quantified. A mixture of H_2O:MeOH:CH_3CN = 171:9:10 was used as a mobile phase at a flow rate of 1 mL/min. The chromatogram was monitored at a wavelength of 259 nm for adenosine quantification.

A total of 20 µL of adenosine deaminase (50 U/mL) was added to a solution of adenosine in 100 mM phosphate buffer (pH 7.5) to transform it into inosine.[13] The resulting inosine was analyzed under the same conditions as those of adenosine at a wavelength of 247 nm. Adenosine and inosine retention times were 6.3 min and 3.4 min, respectively. When this method was applied to the methanol fraction obtained by Amberlite XAD-2 fractionation, the concentration of the fraction was fixed at 10 mg/mL.

Statistical Analysis To investigate the relationship between the adenosine contents in strawberries and antiplatelet activity, the obtained data were analyzed using IBM SPSS v29.0 software (IBM Corp., Armonk, NY, USA). The normality of the data distribution was checked by the Shapiro–Wilk test, and parametrical correlation according the Pearson´s correlation coefficient was calculated.

RESULTS AND DISCUSSION

Prior to an exploration study of antiplatelet compounds in strawberries, we evaluated antiplatelet activity of four cultivars of strawberries. Figure 1 shows antiplatelet activity of the cultivars and ‘Minomusume’ cultivar was selected as the material in this study.

Frozen fruits of ‘Minomusume’ strawberry were thawed and squeezed with a filter cloth, and crude juice was centrifuged to remove solids. Amberlite XAD-2 resin was added to the resulting juice, and a non-adsorbed fraction, a methanol fraction, and an acetone fraction were obtained by elution with methanol and acetone, respectively. Platelet aggregation inhibitory activity was evaluated for each fraction, and the active fractions were subjected to activity-guided separation, as shown in Fig. 2. The methanol fraction (FA-I-2) had a potent inhibitory activity (Fig. 3). FA-I-2 was further fractionated into four fractions. Among them, FA-II-2 with high potency of inhibition was further submitted for separation by repeated medium pressure liquid chromatography using the ODS gel and HPLC. As a result, FA-VI-3 was the most active fraction for both PRP stimulated by ADP and collagen (Fig. 4A–B). Based on NMR spectra analyses of FA-VI-3, it was identified as adenosine along with HPLC analysis. Furthermore, the antiplatelet activity of commercially available adenosine was examined to confirm that the active compound was as expected. As shown in Figs. 4C and 4D, 10 µM adenosine was revealed to inhibit platelet aggregation. Furthermore, FA-II-1 also exhibited activity as high as that of FA-II-2. It turned to be adenosine with a large amount of sugars. It was concluded with this evidence that the active compound in ‘Minomusume’ was adenosine.

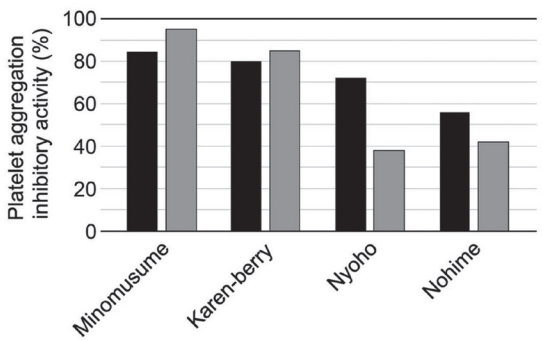

Fig. 1. Antiplatelet Activity of Four Kinds of Popular Strawberries

■ against aggregation stimulated by ADP.
■ against aggregation stimulated by collagen.

As mentioned above, it was found and reported that the platelet aggregation inhibitory activity of strawberry extract varied depending on the cultivar.[8] Since one of the active constituents in the ‘Minomusume’ strawberry was revealed to be adenosine, it was speculated that the potency of the antiplatelet aggregation of each cultivar of strawberry depends on the amount of adenosine contained. Therefore, we quantified the amount of adenosine along with evaluation for platelet aggregation inhibitory activity using ADP and collagen as stimulants of 135 cultivars of strawberries. The correlation between the amount of adenosine and the platelet aggregation inhibitory activity of strawberries was investigated as well.

The extraction method followed the modified method for the ‘Minomusume’ strawberries mentioned above. After adding water to each freeze-dried powdered strawberry to swell, the mixture was centrifuged to obtain the supernatant. Furthermore, Amberlite XAD-2 was added to the supernatant to obtain a fraction containing adenosine, and the fraction eluted with methanol was used in the analysis. The analysis was performed using HPLC equipped with a Develosil C30-UG-5 column using H_2O:MeOH:CH_3CN = 171:9:10 as a mobile phase. Additionally, adenosine deaminase was added to the sample solution before analysis to confirm whether the peak used for quantification was that of adenosine and to separate it from the peaks of contaminated constituents. The resulting inosine from adenosine was also analyzed. The adenosine content in the lyophilized ‘Minomusume’ powder was revealed to be 22.9 ± 6.4 µg/g by this method. However, it did not provide a reliable result when the adenosine content in the methanol eluate of Amberlite XAD-2 resin was less than 1 µg/mL, which corresponds to that in lyophilized powder to be about 1–2 µg/g and a little more though it depends on the weight of the methanol eluate. As a result, only seventeen among 135 cultivars tested gave adenosine content value. Accordingly, adenosine is not a common constituent in strawberry. Furthermore, only fourteen cultivars had a high inhibitory effect against ADP stimulated PRP of 75% or more compared to saline. The correlation between the amount of adenosine and platelet aggregation inhibitory activity of the cultivars whose adenosine content was quantified is shown in Fig. 5. There was a significant positive correlation (0.698, $p < 0.01$) between the amount of adenosine and platelet aggregation inhibitory activity for ADP-stimulated PRP. Similarly, there was a significant positive correlation (0.620, $p < 0.01$) between the adenosine contents and platelet aggregation inhibitory activity for collagen-stimulated PRP. However, some cultivars showed high activity

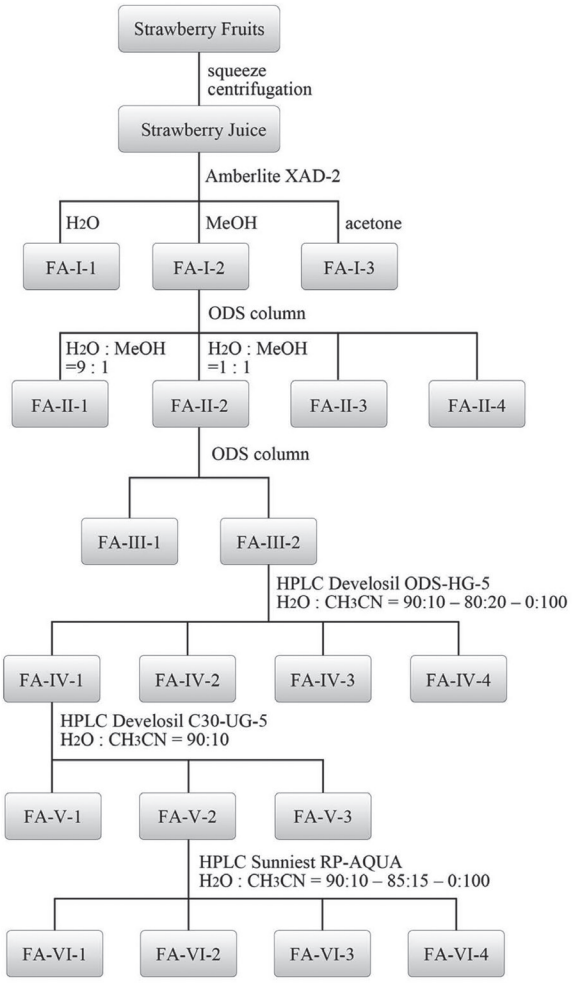

Fig. 2. Separation Scheme of the Active Antiplatelet Compound in 'Minomusume' Strawberries

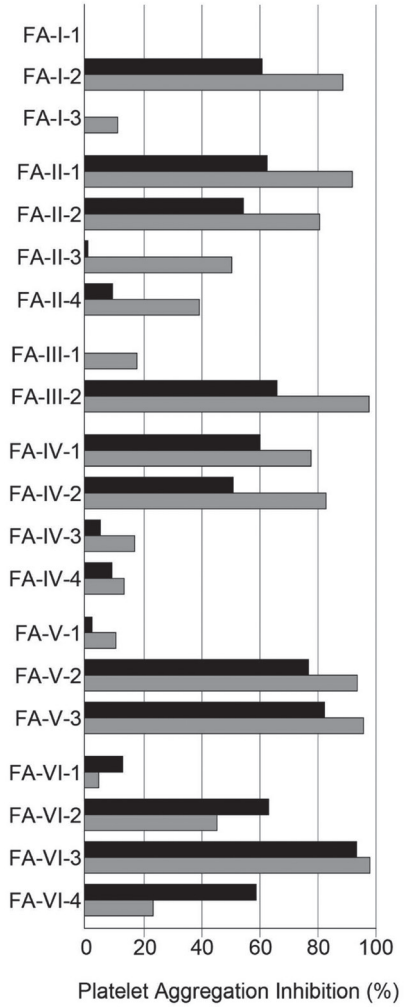

Fig. 3. Antiplatelet Activity of Fractions

against aggregation stimulated by ADP.
against aggregation stimulated by collagen.

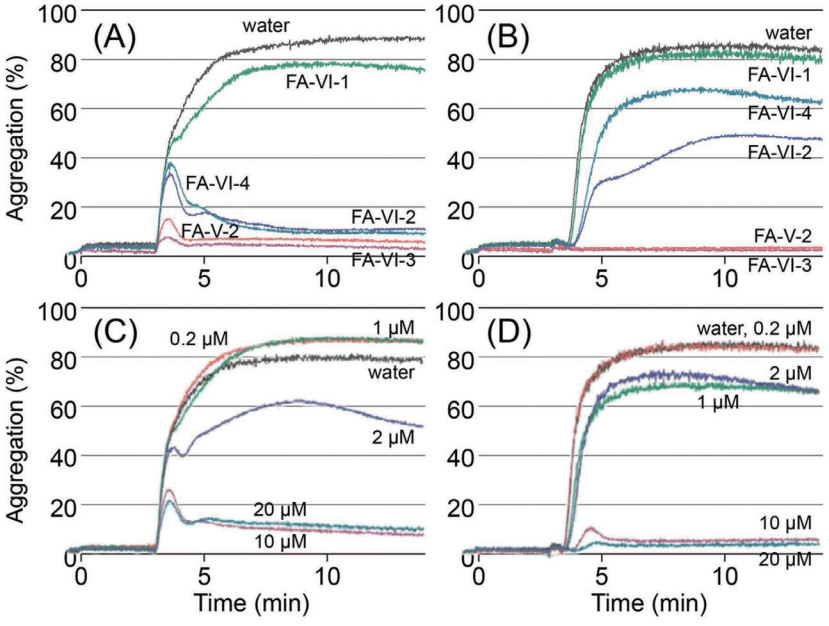

Fig. 4. Effects of Compounds on Platelet Aggregation

(A) Effects of fractions against ADP-stimulated PRP. (B) Effects of fractions against collagen-stimulated PRP. (C) Effects of adenosine at several concentrations against ADP-stimulated PRP. (D) Effects of adenosine at several concentrations against collagen-stimulated PRP.

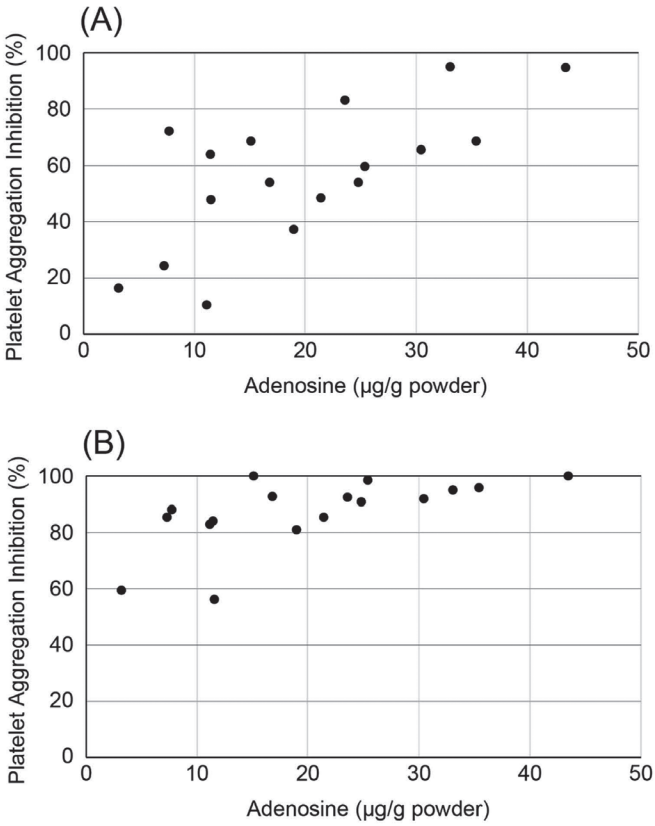

Fig. 5. Correlations between Platelet Aggregation Inhibition and Adenosine Content
(A) ADP-stimulated PRP; (B) Collagen-stimulated PRP.

even though the amount of adenosine was low or not detected. The reasons for these results may include (1) the presence of active compounds other than adenosine, (2) variations in adenosine extraction efficiency to the resin used for sample preparation and (3) fluctuation of the obtained quantification value due to the peaks below or near the quantitative detection limit by HPLC.

CONCLUSION

In this study, adenosine was identified as one of the platelet aggregation-inhibiting compounds in the 'Minomusume' strawberry. The mechanism of action for platelet aggregation of adenosine is well known.[14] In this study, ADP and collagen were used as stimulants to induce platelet aggregation. Collagen increases calcium concentration by binding to glycoprotein (GP) VI and promoting the production of TXA_2. Additionally, ADP inhibits adenylate cyclase (AC) by binding to the ADP receptor and inhibiting the conversion of ATP to cAMP, thereby promoting calcium concentration. As a result, platelets are known to aggregate with fibrinogen, which is calcium dependent. Adenosine binds to the adenosine A_{2A} receptor and activates AC to produce cAMP, suppressing calcium concentration and inhibiting platelet aggregation. It is well known that this mechanism is caused by endogenous adenosine. Still, it was found in this study that the external adenosine obtained from strawberries inhibited aggregation. Due to this mechanism, adenosine is expected to efficiently inhibit thrombus formation in blood vessels.

As mentioned above, there were many cultivars in which the amount of adenosine was below the quantitative detection limit, though some of them showed the higher activity. This result can be accounted that those cultivars contain active compounds other than adenosine along with some defects on the method of quantification. So our following study on strawberry is to explore other antiplatelet active compounds in strawberries. We are also currently investigating a quantitative NMR (qNMR) to quantify adenosine in strawberries. With this method, quantification can be conducted without pretreatment such as fractionation or extraction, so the stability of the data is expected to increase. We will survey the amount of adenosine again by qNMR and examine the correlation with activity.

In addition to what strawberries were revealed to have antithrombotic effects, other activities have also been reported, such as suppressing blood glucose levels[15] and improving brain function.[16] Therefore, considering the high palatability and diversity of intake manners, strawberries can be suitable for preventing lifestyle-related diseases.

Furthermore, it is known that adenosine, identified as an antithrombotic compound in this study, is contained in strawberries and various other vegetables and fruits.[12] Accordingly, we would like to improve the accuracy of the adenosine quantification method, quantify adenosine contained in many different foods, and disseminate information on its effectiveness as an antithrombotic food.

Conflict of interest The authors declare no conflict of interest.

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