Antihyperglycemic Effect of Polyphenols from Acerola (*Malpighia emarginata* DC.) Fruit

Takayuki Hanamura,1,1 Chisato Mayama,1 Hitoshi Aoki,1
Yasushi Hirayama,2 and Makoto Shimizu3

1Research and Development Division, Nichirei Foods Inc., 9 Shinminato, Mihama-ku, Chiba-shi, Chiba 261-8545, Japan
2Quality Assurance Division, Nichirei Inc., 9 Shinminato, Mihama-ku, Chiba-shi, Chiba 261-8545, Japan
3Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Science, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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A crude acerola polyphenol fraction (C-AP) was prepared by subjecting an acerola extract to a C18 cartridge column, and eluting the adsorbed fraction with ethanol containing 10% of acetic acid. C-AP appeared in a previous study to have an inhibitory effect on *α*-glucosidase and particularly on maltase activities. To elucidate the antihyperglycemic effect of C-AP further, we examined the regulation by C-AP of glucose uptake in Caco-2 cell; this resulted in the inhibition of glucose uptake. We next conducted single administration tests of glucose and maltose to ICR mice to investigate whether C-AP really controlled the intestinal glucose absorption in an animal body. The results showed that C-AP significantly suppressed the plasma glucose level after administering both glucose and maltose, suggesting that C-AP had a preventive effect on hyperglycemia in the postprandial state. The mechanism for this effect is considered to have been both suppression of the intestinal glucose transport and the inhibition of *α*-glucosidase. Despite such a preventive effect, the therapeutic effect of C-AP on hyperglycemia appeared to be low from the experiment with KKAy mice.

Key words: acerola; diabetes; Caco-2 cell; *α*-glucosidase; hyperglycemia

Acerola is a fruit found in the region from Central America to northern South America. This fruit is well known to be one of the best natural sources of vitamin C, and has become extremely popular in daily life among people who are health-conscious. Recent studies have shown that an acerola fruit extract may have anticarcinogenic activity against lung cancer,1 an inhibitory effect on NO production,2 and both tumor-specific cytotoxic activity and multidrug resistance reversal activity.3 However, these functions of acerola fruit are presumably due to the effect not only of vitamin C but also of other functional constituents. Concerning these other functional constituents, acerola is known to contain carotenoids1 and polyphenols.4 We have recently isolated three polyphenols from acerola fruit: cyanidin-3-*α*-O-rhamnoside (C3R) and pelargonidin-3-*α*-O-rhamnoside (P3R) as anthocyanins, and quercetin-3-*α*-O-rhamnoside (quercitrin). These acerola polyphenols (APs) were found to have radical scavenging activities and inhibitory effects on both *α*-glucosidase and advanced glycation endproduct (AGE) production which are both closely related to diabetes mellitus and its complications.4

Diabetes mellitus is characterized by chronic hyperglycemia which is associated with an increased risk of cardiovascular diseases.5 Thus, effective management of diabetes mellitus, in particular non-insulin-dependent diabetes mellitus (NIDDM), is to prevent an excess postprandial rise in the blood glucose level or to improve insulin resistance. One of the most effective ways to control the postprandial blood glucose level is to inhibit *α*-amylase or *α*-glucosidase, and therefore a number of inhibitors of these enzymes have been developed.6–9 In a previous study, APs were shown to have inhibitory effects on *α*-glucosidase *in vitro*.4 To further examine the regulatory effect of APs on the postprandial blood glucose level, we investigated whether APs could regulate intestinal glucose transport, because the inhibition of intestinal glucose transport may also participate in decreasing the blood glucose level.10,11 Caco-2 cells have been extensively used to...
investigate the intestinal nutrient transport in vitro. Caco-2 is a human colon adenocarcinoma cell line which spontaneously exhibits various enterocytic characteristics; e.g., the expression of brush border enzymes and nutrient transporters, and the formation of the intercellular tight junction. Since many transporters normally present in the small intestine have been observed in Caco-2 cells, this cell line is considered to be a good model for intestinal epithelial cells. We describe here the inhibitory effect of APs on the intestinal glucose uptake by using Caco-2 cells.

Furthermore, we orally administered maltose and glucose to ICR mice, and measured the effect on the postprandial plasma glucose level after orally administering APs. We also evaluated in a separate study the antihyperglycemic effect of a daily intake of APs by using KKAy mice, a strain possessing features closely resembling those of Type 2 diabetes mellitus and diabetes.15)

Materials and Methods

Samples. Acerola (*Malpighia emarginata* DC.) provided by Nichirei do Brazil Agricola Ltda. (Recife, Brazil) was immediately frozen and stored at −35 °C until needed.

Preparation of the crude acerola polyphenol fraction (C-AP). Frozen acerola fruit (1,000 g) was defrosted, and the seeds were removed. The edible portion of the fruit was homogenized with ethanol (2-liter) and extracted while stirring at room temperature for 1 h to obtain APs. This procedure was repeated twice more. The resulting suspension was filtered, and the filtrate was concentrated and dried under reduced pressure. The resulting AP-powder was dissolved in distilled water to 300 mL and subjected to a conditioned C18 cartridge column (Sep-Pak Vac; Nihon Waters, Tokyo, Japan). The column was washed with ethanol containing 10% of acetic acid, and the eluate was collected in 100-ml fractions. The collected column fractions were pooled and freeze-dried to obtain a crude acerola polyphenol fraction (C-AP; 451 mg) powder which contained 40% of polyphenols as assayed by the Folin-Denis method.16)

Isolation and quantification of the anthocyanins. The procedure for isolating the anthocyanins from acerola fruit has been previously described. Briefly, the edible portion of acerola fruit was homogenized with methanol and extracted while stirring at room temperature. The resulting extract was dissolved in distilled water, and then chromatographed in a C18 cartridge column (Sep-Pak Vac; Nihon Waters, Tokyo, Japan). The adsorbed extract was eluted with 20% methanol containing 0.2% of trifluoroacetic acid (TFA). The anthocyanins were isolated from this extract by HPLC in a 10 × 250 mm C30 column (Develosil RPAQUEOUS-AR-5; Nomura Chemical, Aichi, Japan). The samples were eluted with 20% acetonitrile containing 0.1% TFA at a flow rate of 2.3 ml/min. The isolated anthocyanins were subjected to HPLC, using a LUNA 5 μ C18 (2) column (10 × 250 mm; Phenomenex, Torrance, CA, USA) and purified further. The samples were eluted with 43% methanol containing 0.1% TFA at a flow rate of 2.0 ml/min. The structures of the two isolated anthocyanins (C3R and P3R) are shown in Fig. 1.

The analysis of these anthocyanins was carried out by HPLC (SCL-10Amp system; Shimadzu, Kyoto, Japan), using an XTerra Phenyl 5 μ column (4.6 × 150 mm; Nihon Waters, Tokyo, Japan) eluted with 13% acetonitrile containing 0.1% TFA at a flow rate of 0.8 ml/min. The anthocyanins were quantified from the areas of their peaks recorded at 500 nm, using standard solutions of isolated C3R and P3R.

Cell culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The growth medium contained Dulbecco’s modified Eagle’s medium (Nissui Pharmaceuticals, Tokyo, Japan) with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA). The growth medium contained Dulbecco’s modified Eagle’s medium (Nissui Pharmaceuticals, Tokyo, Japan) with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA). The monolayer cultures were grown in a humidified atmosphere containing 5% CO₂.

Confluent monolayers were subcultured every 3 or 4 days and harvested with 0.25% trypsin plus 0.8 mM EDTA, resuspended, and finally seeded in a new culture dish (1.4 × 10⁵ cells/dish).

Measurements of the glucose uptake. The Caco-2 cells were seeded 14–17 days before their use. The monolayer cells were rinsed twice with 700 μl of Hank’s balanced buffer solution (HBSS). An HBSS solution containing 123 mM of 3-O-[methyl-³H]-D-glucose (3-O-met glucose; Amersham, Buckinghamshire, UK) and C-AP or purified APs was then added to the cell monolayers in the presence of an uptake buffer (140 mM NaCl, 5.4 mM KCl, 1.26 mM CaCl₂, 0.81 mM MgSO₄, 0.44 mM KH₂PO₄, and 5.5 mM mannitol at pH 7.4). After incubating for 20 min at 37 °C, the solution was removed, and each monolayer was washed twice with
ice-cold PBS containing 0.05% sodium azide. The monolayers were then solubilized with 0.1% Triton X-100, and the radioactivity in each monolayer was determined with an LSC 5100 liquid scintillation analyzer (Aloka, Tokyo, Japan).

**Single administration test.** Male ICR strain mice (Clea, Tokyo, Japan) weighing 25–30 g (6 weeks of age) were fed on a normal commercial diet (MF; Oriental Co., Tokyo, Japan) for one week. The room temperature was controlled at 25 ± 1 °C, and lighting was on a 12-h cycle (0600–1800: light).

The mice were divided into 2 groups (n = 15 for the glucose administration experiment and n = 12 for the maltose one) and starved overnight. A 43.75 mg/ml of control was administered in 200-μl doses (for 35 g of weight) to the C-AP-treated group. To the control group was administered PBS instead of the C-AP solution. A 350 mg/ml amount of the glucose or maltose solution was continuously administered orally in 200-μl (for 35 g of weight) to all the mice. Before and 30, 60 and 90 min after this administration, blood samples were taken from the tail vein. All the samples were collected with a heparin-coated capillary and centrifuged (10,500 × g for 5 min) to separate the plasma. The plasma samples were frozen at −30 °C until being assayed. The plasma glucose concentration was measured with a Glucose CII Test kit (Wako Chemicals, Osaka, Japan).

**Evaluation of the antihyperglycemic effect of the daily intake.** Male KKAy strain mice (Clea, Tokyo, Japan) weighing 20–25 g (5 weeks of age) were fed on a normal commercial diet (MF) for one week. The room temperature was controlled at 25 ± 1 °C, and lighting was on a 12-h cycle (0600–1800: light).

The mice were divided into 2 groups: the control group (n = 5) was given distilled water to drink, while the C-AP-treated group (n = 6) received 1 mg/ml of a C-AP solution instead of distilled water to drink for a period of 8 weeks with free access. C-AP was dissolved in distilled water and passed through a polycarbonate filter (0.4 μm, Transwell; Costar Co., Cambridge, MA, USA). The plasma glucose levels under non-fasted and fasted conditions, HbA1c level and insulin level were measured 0, 2, 5 and 8 weeks after starting the C-AP administration. A blood sample for the non-fasted blood glucose and insulin level measurements was collected, and thereafter, the mice were fasted more than 20 h. The next day after fasting, a blood sample for the fasted blood glucose and HbA1c level measurement was collected. For the measurements of the blood glucose and insulin levels, blood samples collected with a heparin-coated capillary from the tail vein of the mice were centrifuged at 10,500 × g for 5 min, and the resulting plasma samples were frozen at −30 °C until being assayed. For the HbA1c measurement, whole blood samples were used immediately after collecting.

Measurement of the plasma glucose level was performed as described in the previous section. The HbA1c level was measured with a DCA2000 system (Bayer Sankyo, Tokyo, Japan), and the insulin level was determined with an enzyme-linked immunosorbent assay insulin kit (Morinaga, Tokyo, Japan). After the C-AP-treated period of 8 weeks, the insulin resistance of the KKAy mice and ICR mice (for the normal level) was measured. The plasma glucose level was monitored 15, 30 and 60 min after an intraperitoneal application to the mice of one unit/kg of insulin to measure the insulin resistance.

**Statistical analysis.** A statistical evaluation was made by Student’s t-test. Differences were considered to be statistically significant when p < 0.05. Each data value is expressed as the mean ± SD.

**Results**

**Measurement of the glucose uptake**

To study the effects of APs on the intestinal glucose absorption, we examined the level of glucose uptake by Caco-2 cells after treating the cells with various doses of C-AP. As shown in Fig. 2A, the glucose uptake level was decreased in a dose-dependent manner by adding C-AP, with an IC50 value of approximately 0.2 mg/ml.

The inhibitory effects of C3R and P3R, which had been isolated from C-AP, on the glucose uptake by Caco-2 cells were also studied. It was found that the inhibitory effect of P3R was higher than that of C-AP, whereas C3R showed almost the same level of inhibition as C-AP (Fig. 2B). The C3R and P3R contents in C-AP were 10.1% and 1.04%, respectively.

**Single-administration test**

The plasma glucose level increased sharply in both groups of mice, reaching the maximum within 30 min after oral administering glucose or maltose. However, in the glucose administration test, the plasma glucose level of the C-AP-treated mice was significantly lower than that of the control group, this effect being continued throughout the experimental period, suggesting that C-AP suppressed the intestinal glucose absorption (Fig. 3). In the maltose administration test, C-AP also significantly suppressed the plasma glucose level for up to 30 min after the maltose administration. However, the lower plasma glucose level in the C-AP-treated mice did not last for 90 min (Fig. 4).

**Antihyperglycemic effect of a daily intake of C-AP**

The antihyperglycemic effects of a chronic C-AP treatment were investigated with KKAy mice. The body weight and food intake of individual mice were monitored. The body weight increased from 21.7 ± 0.4 g to 42.2 ± 2.0 g for the control mice and from 21.3 ± 0.6 g to 42.0 ± 2.8 g for the C-AP treated mice, while the food intake varied within 4.1 to 7.3 g/day.
during the experimental period. There was no significant difference between C-AP treated and control mice in either parameter throughout the experimental period.

The time-course characteristics of the plasma glucose level of the C-AP-treated and control mice under the non-fasted and fasted conditions are shown in Fig. 5. No significant difference in the plasma glucose level was apparent between the two groups of fasted mice (Fig. 5A), whereas the C-AP-treated mice appeared to have a significantly lower plasma glucose level until the 5th week after C-AP administration when nonfasted (Fig. 5B). Similarly, C-AP tended to suppress the blood HbA1c level, although the difference from the control mice was not statistically significant ($p > 0.05$; Fig. 6).

On the other hand, C-AP had no effect on the insulin resistance of the KKAy mice (Fig. 7). The plasma insulin level fluctuated within 13 to 25 pg/ml of plasma in both groups during the experimental period, and there was also no significant difference between the two groups (data not shown).

**Discussion**

Polyphenol compounds extracted from acerola fruit have appeared to show an inhibitory effect on α-glucosidase and particularly on maltase activities.\(^4\) Inhibiting the activity of α-glucosidase or amylase can prevent intestinal glucose absorption and suppress the blood glucose level in the postprandial state. However,
inhibitors of these enzymes are not able to prevent glucose absorption when glucose itself has been ingested. Among foodstuffs from natural sources, there are a number that contain glucose as sugar. Therefore, to control the postprandial blood glucose level, it might be important to block intestinal glucose absorption itself in addition to inhibiting the glucosidase or amylase activity.

To investigate the intestinal glucose absorption in vitro, we evaluated the glucose uptake by using Caco-2 cells and demonstrated C-AP to be a good inhibitor of glucose uptake by these cells (Fig. 2A). C3R and P3R are considered to be the constituents that contributed to this effect, although the role of other constituents in C-AP cannot be ruled out (Fig. 2B). It was found that P3R more effectively inhibited the glucose uptake than C3R. These two anthocyanins differ in their structures by the number of OH-bonds of the B-ring in polyphenol (Fig. 1). Such a minor structural difference might be responsible for the observed difference in their inhibitory activities against glucose uptake, although the precise mechanism is still unclear.

With regard to the intestinal absorption of nutrients it is important to consider the transport mechanism. The components which regulate intestinal glucose absorption probably act on such glucose transporters as SGLT1 and GLUTs. It is known that the sodium-dependent glucose transporter, SGLT1, is the most abundant glucose transporter in the small intestine. Caco-2 cells have been reported to express such glucose transporters as SGLT1 and GLUTs. However, in view of the fact that there are considerable differences in the properties of Caco-2 cells in different laboratories, probably because of their heterogeneous nature, the expression level of SGLT1 also varies in different laboratories. It is therefore possible that the Caco-2 cells used in this study had a low expression level of SGLT1, and were not appropriate as intestinal model cells. It consequently seems to be insufficient to evaluate the regulatory effects of C-AP on the intestinal glucose absorption level merely by using Caco-2 cells.

This prompted us to further substantiate the in vitro results of C-AP activities by the effect on actual animal bodies. In a single administration test using ICR mice, we investigated whether C-AP could really control the intestinal glucose absorption in actual animal bodies, and if so, which pathway C-AP blocked, carbohydrate degradation by α-glucosidase or intestinal glucose absorption. We conducted maltose and glucose administration tests to do this, and the results show that C-AP significantly suppressed the plasma glucose level after an oral administration of both maltose and glucose (Figs. 3 and 4). The fact that C-AP suppressed the postprandial blood glucose level, even when glucose itself was administered, in addition to the results of in vitro studies, substantiated the mechanism for C-AP
mediated suppression of the postprandial blood glucose level being both the suppression of intestinal glucose transport and the inhibition of $\alpha$-glucosidase (Fig. 8).

It has been demonstrated that C3R and P3R, which are the main anthocyanins contained in C-AP, had an inhibitory effect on both the $\alpha$-glucosidase activity and intestinal glucose uptake from the in vitro study just described. Therefore, these anthocyanins would probably participate in the C-AP-induced regulation of intestinal glucose absorption. However, the contents of C3R and P3R in C-AP are no more than 10.1% and 1.04%, respectively. Therefore, it was necessary to investigate the role of other functional constituents in the acerola extract to better elucidate the mechanism underlying suppression of the postprandial blood glucose level by C-AP.

Since C-AP had been found to lower the postprandial blood glucose level by a single oral administration, we tried to investigate whether C-AP had a therapeutic effect on hyperglycemia as well. We used KKAY mice for this purpose, an animal model for type 2 diabetes mellitus. These mice are known for genetically induced diabetes,24) and consequently had hyperinsulinemia. While the C-AP-treated KKAY mice showed a lower plasma glucose level than the control mice in the non-fasted condition and tended to suppress the blood HbA1c level, C-AP had no effect on the plasma glucose level under the fasted condition, nor on the plasma insulin level and the insulin resistance (see Figs. 5–7).

The chronic hyperglycemia observed with type 2 diabetes mellitus is caused mainly by insulin resistance, the disorder being characterized by the impairment of either glucose-induced insulin secretion from pancreatic $\beta$-cells, or insulin-induced glucose uptake in adipose cells or skeletal cells.25) Thus, to ameliorate the hyperglycemia seen in the KKAY mice, improving the insulin sensitivity would be more necessary than suppressing the glucose absorption from intestinal cells. It has recently been reported that anthocyanins present in fruits stimulated insulin secretion in pancreatic $\beta$-cells.26) In the present study, however, C-AP appears to have had no effect on insulin resistance, despite containing anthocyanins. This could be related to the bioavailability and metabolism of ingested C-AP that seem to play crucial roles in insulin resistance. In fact, it has been reported that the bioavailability of anthocyanins was rather low,27,28) suggesting that the low bioavailability of C-AP could not elicit the insulin secretagogue in the present study. It seems essential, therefore, to examine the bioavailability and metabolism of the constituents of C-AP and a mechanism to increase their availability.

The C-AP treated KKAY mice, however, transiently showed a lower plasma glucose level than the control mice in the non-fasted condition (Fig. 5B). The reason for this phenomenon is unclear so far. However, as already described, C-AP had the effect of lowering the postprandial blood glucose level, although it had no effect on insulin resistance. We therefore suspect that C-AP acted to delay the impairment of diabetes mellitus by decreasing the state of hyperglycemia. The precise mechanism for this phenomenon is now under investigation.

In conclusion, we have demonstrated that C-AP had a preventive effect on hyperglycemia in the postprandial state, the mechanism for this effect being considered to involve both the suppression of intestinal glucose transport and the inhibition of $\alpha$-glucosidase.
transport and the inhibition of α-glucosidase. In the case of KKAy mice, on the other hand, the therapeutic effect of C-AP on hyperglycemia was insignificant.

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