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Chestnut Astringent Skin Extract, an α-Amylase Inhibitor, Retards Carbohydrate Absorption in Rats and Humans

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Summary Inhibitors of carbohydrate-hydrolyzing enzyme play an important role to control postprandial blood glucose levels. In this paper, we investigated the effect of an ethanol extract from chestnut astringent skin (CAS) on alpha-amylase. Chestnut astringent skin extract strongly inhibited human and porcine pancreatic alpha-amylase. We also investigated the effect of CAS extract on carbohydrate absorption in rats and humans. Oral administration of CAS extract to normal rats fed corn starch (2 g/kg body weight), significantly suppressed the increase of blood glucose levels after starch loading in a dose-dependent manner. The effective dose of CAS extract required to achieve 20 and 40% suppression of the rise in blood glucose level was estimated to be 40 and 155 mg/kg body weight, respectively. Chestnut astringent skin extract also suppressed the rise in plasma insulin level and the fall in plasma non-esterified fatty acid level. In the type 2 diabetic rat model, CAS extract significantly suppressed the rise in blood glucose level after starch loading in a dose-dependent manner. Chestnut astringent skin extract also suppressed the rise in plasma glucose level after boiled rice loading in a dose-dependent manner in humans. The amount of CAS extract required to achieve 11 and 23% suppression in the rise in plasma glucose level was 300 and 600 mg/person, respectively. These results suggest that CAS extract retards absorption of carbohydrate and reduces post-prandial hyperglycemia.

Key Words chestnut astringent skin, amylase inhibitor, blood glucose, oral carbohydrate tolerance test

The prevalence of diabetes is increasing worldwide due to changes in modern lifestyles (*1*, *2*). Most diabetic patients suffer from type 2 diabetes which is closely related to obesity (*3*, *4*). Diet and exercise therapy are key factors for preventing and treating type 2 diabetes. Maintenance of healthy blood glucose levels is of particular importance and is greatly affected by dietary polysaccharides such as starches and glycogen. These polysaccharides are broken down by digestive enzymes such as α-amylase, a key enzyme in polysaccharide digestion. α-Amylase catalyzes the first step in the digestion of polysaccharides, hydrolyzing the α-1,4-glucoside linkages. The amylases are contained in the saliva and pancreatic fluid of mammals and transform polysaccharides into oligosaccharides in the alimentary canal. Control of α-amylase activity is important in order to inhibit excess energy supply and to regulate blood glucose levels.

Inhibitors of α-amylase derived from various sources have been reported and their effects have been investigated. Ali et al. reported that extracts of six selected Malaysian plants strongly inhibited α-amylase activity (*5*). Tormo et al. reported that α-amylase inhibitor from white beans reduced glycemia in both non-diabetic and diabetic animals and reduced the intake of food and water (*6*). DiMagno et al. reported that an α-amylase inhibitor from wheat might be useful for treatment of type 2 diabetes mellitus (*7–9*).

In the screening of inhibitors of carbohydrate-hydrolyzing enzyme from plant, we discovered CAS inhibited α-amylase. The chestnut is a deciduous timber tree of the genus *Castanea*, in the beech family. Native chestnuts are distributed in the temperate regions of the Northern Hemisphere. They bear burrlike fruits that contain two or three edible nuts. The nuts are important as food and are exported in large quantities. They are cultivated over large area of East Asia such as China, Korea and Japan. We investigated the potential dietary use of CAS as an α-amylase inhibitor because industrial CAS-free pea processing of chestnuts generates a significant quantity of CAS as a chestnut waste material. In this paper, we report that CAS extract is a strong inhibitor of α-amylase and may retard absorption of carbohydrates and reduce post-prandial hyperglycemia in rats and humans.

MATERIALS AND METHOD

Materials. α-Amylases from porcine pancreas, human pancreas and human saliva were obtained from

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Abbreviations: AUC, area under the curve; CAS, chestnut astringent skin; IC_{50}, concentration of inhibitor to inhibit 50% of its activity; NEFA, non-esterified fatty acid.

Sigma-Aldrich Japan (Tokyo, Japan). Soluble starch was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dried chestnut astringent skin (CAS) powder and raw CAS were obtained from Chuon Co. Ltd. (Matsuyama, Japan).

Preparation of CAS extract. One hundred grams of the dried CAS powder were added to 2 L of 50% v/v aqueous acetonitrile, followed by stirring at 37°C for 12 h. The mixture was filtered and the filtrate was concentrated and lyophilized, to generate CAS extract powder, designated CAS-ex1. A large-scale extract was prepared from raw CAS to mimic industrial-scale production. One thousand kilograms of raw CAS was added to 2,000 kg of 75% aqueous ethanol and subjected to the same procedure described above. The large-scale CAS extract powder was designated CAS-ex2.

Assay methods. α-Amylase activity was determined by measuring the reducing power of released oligosaccharide from soluble starch by the method of Miller (*10*) with the following minor modifications. The assay system comprised the following components in a total volume of 1 mL: 100 mM sodium phosphate, pH 6.8, 17 mM NaCl, 5 mg soluble starch, 100 μL of inhibitor solution, and 10 μL of enzyme solution. After incubation at 37°C for 30 min, the reaction was stopped by addition of 0.1 mL 2 N NaOH and 0.1 mL color reagent (4.4 μmol of 3,5-dinitrosalicylic acid, 106 μmol of potassium sodium (+)-tartrate tetrahydrate and 40 μmol of NaOH), followed by a 5-min incubation, at 100°C and subsequent A_{540} measurement.

Polyphenols (total phenolics) in the CAS extract were determined by the method of Folin-Denis (*11*, *12*).

Oral carbohydrate tolerance test in rats. The experimental animal protocol was approved by the Animal Study Committee of Ehime University. Male Wistar King rats, weighing 190–230 g were starved overnight (15 h), and divided into two groups. The test group received 2 mL of corn starch suspension (2 g/kg body weight) containing 1 mL of CAS extract powder solution while the control group received corn starch suspension and 1 mL of water. After administration, blood samples were collected from the tail vein or artery at regular intervals. Blood glucose was measured using a blood glucose test meter, GLUCOCARD (Arkray Inc., Kyoto, Japan). After a 1-wk interval, the test and control groups were switched and the experiment was repeated.

Type II diabetic model rats (GK/jcl) obtained from CLEA Japan, Inc. (Tokyo, Japan), weighing 310–360 g were starved overnight (15 h), and subjected to the carbohydrate tolerance test as described above.

Plasma insulin and free fatty acids were assayed in normal rats subjected to the carbohydrate tolerance test as follows. Male Wistar King rats, weighing 190–230 g were starved overnight (15 h) and divided into three groups. The test group received 2 mL of corn starch suspension (2 g/kg body weight) containing 1 mL of CAS extract powder solution (300 mg/kg body weight). Control group 1 received corn starch suspension (2 g/kg body weight) and 1 mL water and control group 2 received only water. After administration, blood samples were collected from the inferior vena cava using a heparinized syringe at regular intervals, and centrifuged immediately at 2,000 $\times g$ for 5 min. Plasma glucose, insulin and non-esterified fatty acids (NEFA) were determined using the Glucose C-II test Wako (Wako Pure Chemical Industries, Ltd.), the Morinaga insulin assay kit (Morinaga Institute of Biological Science Inc., Yokohama, Japan), and the NEFA C-test Wako (Wako Pure Chemical Industries, Ltd.), respectively.

Oral carbohydrate tolerance test in human subjects. The human study protocol was approved by the Ethics Committee of Kimura Hospital and was carried out in Genki Plaza Medical Center for Healthcare (Tokyo, Japan), in accordance with the principles of the Helsinki Declaration as revised in 2000. All subjects gave informed consent. Eleven healthy Japanese volunteers (male : female=8 : 3; age 30–59 y (average age 46.6±9.3 y; fasting blood glucose level <126 mg/dL; HbA1c <6.5%) were starved overnight. Studies were done on three separate mornings at least 3 d apart. The first day, subjects ingested 200 g of boiled rice and 200 mL of water and venous blood samples were taken for determination of plasma glucose level before, and 30, 60, 90 and 120 min after eating. The second day, subjects ingested 200 g of boiled rice and 200 mL of water containing 300 mg of CAS extract. On the third day, they ingested 200 g of boiled rice and 200 mL of water containing 600 mg of CAS extract, after which blood samples were taken. The blood samples were centrifuged immediately at 2,000 $\times g$ for 5 min and plasma glucose was determined.

Statistical analysis. Results are expressed as the mean±SE. The statistical significance of differences with and without (control) CAS extract was assessed using the paired Student's *t*-test.

RESULTS

CAS extract powder (CAS-ex1) is a bitter-tasting dark brown powder. It was found to strongly inhibit pig pancreatic α-amylase activity. A concentration of 13.1 μg/mL was determined to result in 50% inhibition. Because CAS extract was found to inhibit mammalian α-amylase, we examined whether the CAS extract could exert an inhibitory effect on blood glucose level increase after starch loading in rats that had fasted 15 h. Blood glucose levels increased from a baseline of 52.7±1.83 mg/dL at 0 min to a peak of 113.6±3.86 mg/dL (increased blood glucose value 60.9±3.9 mg/dL) at 60 min after starch administration (2 g/kg body weight). The rise in blood glucose was suppressed in a dose-dependent manner when CAS extract (10, 25, 50, 100 and 300 mg/kg body weight) was given with starch (Fig. 1A). The area under the curve ($AUC_{0-180\,min}$) for CAS extract administration was also found to decrease with the increase in CAS extract administration in a dose-dependent manner compared to administration of starch alone (Fig. 1B). Figure 1C shows % of AUC change for CAS extract administration relative to administration of starch alone. Based on the observed relationship between

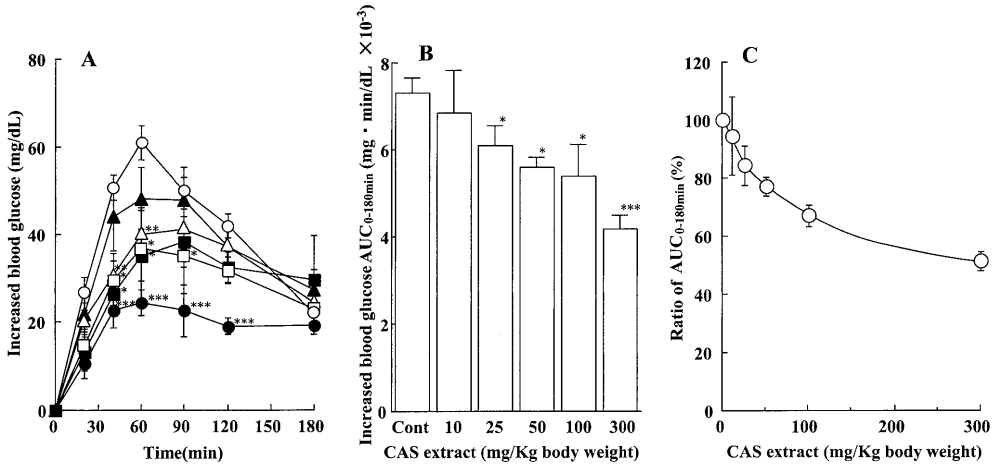

Fig. 1. Effects of CAS extract (CAS-ex1) on increased blood glucose concentrations (A), the area under the curve (AUC) values (B), and ratio of AUC (C) in normal rats. A: Rats fasted for 15 h, and CAS extract (10 (▲), 25 (△), 50 (■), 100 (□) and 300 (●) mg/kg body weight) and starch (2 g/kg body weight) were administered. As a control, rats were given only starch (2 g/kg body weight) (○). B: $AUC_{0-180\,min}$ is the area under the curve of the incremental blood glucose level up to 180 min. C: The ratio (%) of $AUC_{0-180\,min}$ for CAS extract against $AUC_{0-180\,min}$ for the control was used to estimate 20 and 40% suppression values. Results are expressed as means±SE. $n=8$. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. control.

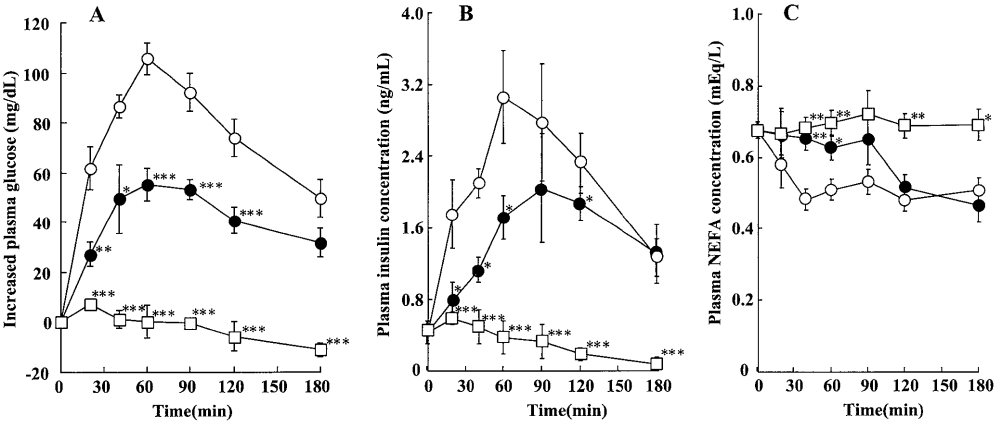

Fig. 2. Effects of CAS extract (CAS-ex1) on increased plasma glucose (A), insulin (B), and NEFA (C) concentrations in normal rats. Rats fasted for 15 h, and CAS extract (300 mg/kg body weight) and starch (2 g/kg body weight) were administered (●). As a control, rats were given only starch (2 g/kg body weight) (control 1, ○) or only water (control 2, □). The results are expressed as means±SE. $n=8$. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. control 1.

the amount of CAS extract and reduction ratio of $AUC_{0-180\,min}$, the effective dose of CAS extract required to achieve 20 and 40% suppression of blood glucose level rise was estimated to be 40 and 155 mg/kg body weight, respectively (Fig. 1C).

For the assay of plasma insulin and NEFA on carbohydrate tolerance test, blood samples were collected from the inferior vena cava of normal rats (Fig. 2). Plasma glucose levels were found to increase from a baseline value of 47.3±0.84 mg/dL at 0 min to a peak of 149.5±6.5 mg/dL (increased plasma glucose value 102.2±6.4 mg/dL) at 60 min after administration of starch (2 g/kg body weight) (Fig. 2A). Plasma glucose levels decreased slightly after administration of water only. A similar pattern was observed in the plasma insulin levels which also increased from a baseline value of 0.432±0.013 ng/mL at 0 min to a peak of 3.07±0.52 ng/mL at 60 min after starch administration (Fig. 2B). Plasma insulin levels also decreased slightly after administration of water only. When a CAS extract (300 mg/kg body weight) was given with starch, the rise in plasma glucose (peak 106.7±6.8 mg/dL, increased 55.2±6.7 mg/dL, at 60 min) and insulin (peak 2.04±0.61 ng/mL at 90 min) levels were suppressed significantly. While plasma NEFA levels were high upon fasting (0.68±0.023 mEq/L at 0 min), they decreased significantly after starch administration (0.48±0.029 mEq/L at 40 min) (Fig. 2C). Plasma NEFA levels remained at a high level (0.69±0.016 mEq/L at 180 min) after administration of water only. The drop in plasma NEFA levels by administration of starch were suppressed significantly by administration of starch with CAS extract. The area under the curve ($AUC_{0-180\,min}$) of plasma glucose rise and plasma insulin level decreased significantly with administration of CAS extract: 45% suppression of plasma glucose rise and 27% suppression of plasma insulin increase. Conversely, $AUC_{0-180\,min}$ of plasma NEFA level increased significantly

(about 12%) with administration of CAS extract (Table 1).

In diabetic rats (GK/jcl), blood glucose levels increased from a baseline of 96±8.08 mg/dL at 0 min to a peak of 406±19.4 mg/dL at 60 min after administration of starch (2 g/kg body weight). The rise of the blood glucose levels were suppressed significantly in a dose-dependent manner when CAS extract was given with starch. Peak increased blood glucose levels were 310±18.1, 256±14.7 and 197±14.2 mg/dL at 60 min for CAS extract administered at doses of 0, 100 and 300 mg/kg body weight, respectively (Fig. 3A). $AUC_{0-180\,min}$ for CAS extract administration also decreased with the increase in CAS extract administration in a dose-dependent manner compared to administration of starch alone (Fig. 3B): 22 and 46% suppression for CAS extract administration of 100 and 300 mg/kg body weight, respectively.

The large-scale extraction was done to mimic industrial production for potential human application. Ethanol was used as an extraction solvent instead of acetonitrile, because the toxicity of ethanol is lower than that of acetonitrile. A typical aqueous ethanolic CAS extract (CAS-ex2) is shown in Table 2. It contains a high concentration of polyphenols, about 20%. Heavy metal content and bacterial contents were very low. The extract was found to inhibit α-amylase activity in a dose-dependent manner with a relatively low 50% inhibition value (IC_{50}). Human pancreatic amylase was strongly inhibited. The IC_{50} value for human pancreatic amylase was about two times lower than that for pig pancreatic amylase (Table 2). Based on the carbohydrate tolerance test in normal rats, 33% suppression of the blood glucose level rise was observed with administration of the CAS extract (CAS-ex2) at 100 mg/kg body weight (data not shown).

Prior to performing the test on human subjects, the CAS extract powder was assayed for subchronic toxicity using SD rats (6 males and 6 females). CAS extract powder (1,000 mg or 2,000 mg/kg body weight/d) was administered to rats via a stomach tube for 28 d. Dur-

Table 1. Effects of CAS extract (CAS-ex1) on increased plasma glucose, plasma insulin and plasma NEFA $AUC_{0-180\,min}$[1] in normal rats.[2]

Group	Glucose (mg×min/dL)	Insulin (ng×min/mL)	NEFA (mEq×min/L)
Control 1	13,000±2,000	369±6.03	94.0±1.70
CAS extract	7,940±1,550***	269±9.77***	105±2.97**
Control 2	−550±1,700***	59.5±2.24***	124±1.44***

[1] $AUC_{0-180\,min}$ is the area under the curve up to 180 min in Fig. 2.
[2] Results are expressed as means±SE. $n=8$. $^{**}p<0.01$ and $^{***}p<0.001$ vs. control 1.

Table 2. Characterization of a typical CAS extract (CAS-ex2).[1]

Parameter	Value
Amylase inhibition (IC_{50})[2]	
Human pancreas	7.5±1.2 μg/mL
Human saliva	9.4±1.3 μg/mL
Porcine pancreas	18.2±3.2 μg/mL
Polyphenol content	20.7±0.53%
Ash content	3.3±0.14%
Water content	7.4±0.64%
Arsenic content	<2 ppm
Lead content	<10 ppm
Live bacteria	<1,000 /g
Live fungi	<100 /g
Colibacillus group	—

[1] The results are expressed as means±SE of six CAS extracts.
[2] The IC_{50} value is defined as the concentration of inhibitor to inhibit 50% of its activity under the assayed conditions.

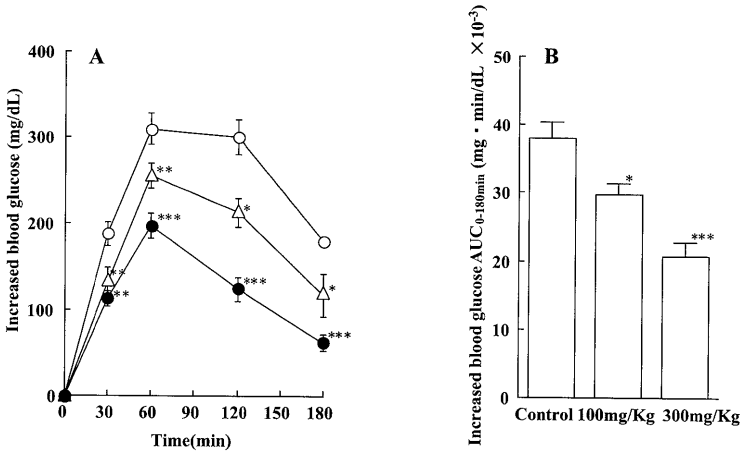

Fig. 3. Effects of CAS extract (CAS-ex1) on increased blood glucose concentrations (A) and $AUC_{0-180\,min}$ (B) in diabetic rats (GK/jcl). Rats fasted for 15 h, and CAS extract (100 mg/kg body weight (△), 300 mg/kg body weight (●) and starch (2 g/kg body weight) were administered. As a control, rats were given only starch (2 g/kg body weight) (control (○). The results are expressed as means±SE. $n=8$. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. control.

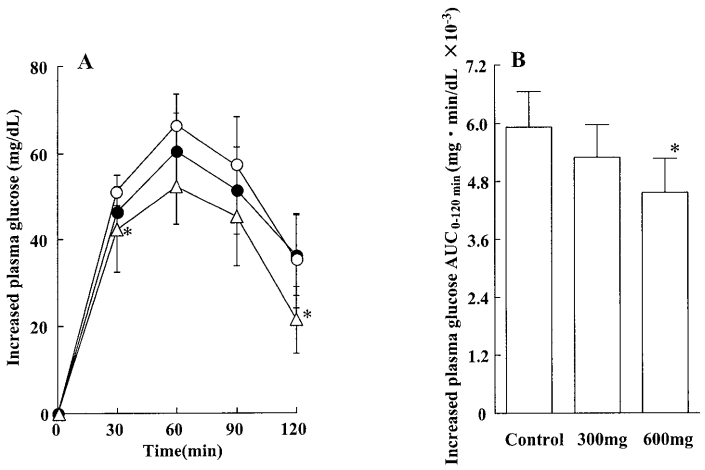

Fig. 4. Effects of CAS extract (CAS-ex2) on increased plasma glucose concentrations (A) and $AUC_{0-120\,min}$ (B) in healthy volunteers. Plasma glucose levels were measured after ingestion of 200 g of boiled rice with CAS extract (300 mg/person (●) and 600 mg/person (△)). As a control, volunteers were given only boiled rice (○). The results are expressed as means±SE. $n=11$. $^*p<0.05$ vs. control.

ing the study, the treatment induced no noticeable effects on clinical signs, survival or ophthalmology. Body weight gain, food intake, water intake, urinalysis, hematology, blood biochemistry, gross pathology and organ weights exhibited no differences of toxicological significance between control and treated rats (data not shown). Thus, these results indicated a no-observed-adverse-effect level of CAS extract to be over 2,000 mg/kg body weight/d. The effect of CAS extract taken with boiled rice (200 g) on plasma glucose level in healthy Japanese volunteers is shown in Fig. 4. Following administration of boiled rice with water (control), plasma glucose levels increased from a baseline of 100.3±5.01 mg/dL at 0 min to a peak of 166.8±11.67 mg/dL at 60 min. The rise in plasma glucose was suppressed in a dose-dependent manner when CAS extract was given with the boiled rice. Peak levels of increased plasma glucose were 66.5±12.1, 60.5±11.2, and 52.3±11.7 mg/dL at 60 min for CAS extract doses of 0, 300, and 600 mg/person, respectively (Fig. 4A). Starch absorption ($AUC_{0-120\,min}$) for administration of CAS extract also decreased with the increase in dose in a dose-dependent manner compared to ingestion of rice alone (Fig. 4B). Suppression by 11 and 23% was observed by CAS extracts administered at doses of 300 and 600 mg/person, respectively.

DISCUSSION

In this paper, we report a potent α-amylase inhibitor(s) in CAS. Many α-amylase inhibitors have been isolated from plants and microorganisms (*13*, *14*). α-Amylase inhibitors are roughly divided into two classes, proteinaceous and non-proteinaceous. We presume that the α-amylase inhibitor(s) in the CAS extract is not proteinaceous, because the inhibitory activity was heat stable; over 90% of the inhibitory activity was maintained following a 1 h incubation at 100°C (data not shown). The inhibitory activity was slightly extracted in water or in hot water (data not shown). Therefore we also presume that the α-amylase inhibitor(s) is not a carbohydrate derivative. The inhibitory activity was extractable in 50% aqueous acetonitrile, a solvent commonly used to extract polyphenols. The inhibitory activity was also extractable in aqueous acetone and aqueous ethanol, but not in 100% acetonitrile, acetone, ethanol or hexane. The CAS extract contained about 20% polyphenolic material (Table 2). Furthermore, when CAS extract powder was re-extracted with aqueous ethanol, the content of polyphenolic material increased about 3-fold (about 60%) and the amylase inhibitory activity also increased about 2.6-fold (data not shown). These results suggest that an active component(s) of the CAS extract may be a polyphenol. Kandra et al. have reported that tannic acid, a polyphenol, exhibited mixed non-competitive type inhibition of α-amylase activity (*15*). We compared the inhibitory activity of tannic acid with that of the CAS extract and found that the former had an IC_{50} toward porcine pancreatic α-amylase of greater than 1 mg/mL, which was over 50 times higher than that of the CAS extract (Table 2). Therefore, we speculate that the major active components of the CAS extract may not include tannic acid. Purification of the active components from the CAS extract could lead to development of an antihyperglycemic drug.

The CAS extract also inhibited α-glucosidase activity of the rat small intestine. However, the IC_{50} values of the CAS extract (CAS-ex2) were 50 to 100 times higher than that for α-amylase: 640 and 940 μg/mL towards maltase and sucrase activity, respectively. The extract also inhibited rat pancreatic lipase activity with an IC_{50} value of 510 μg/mL. Protease inhibitory activity towards trypsin and chymotrypsin was very weak. IC_{50} values were over 2 mg/mL. These results suggest the CAS extract specifically and strongly inhibited α-amylase activity.

When CAS extract was given with starch to the normal rat, the rise in plasma glucose and insulin, and the

fall in plasma NEFA levels were significantly suppressed (Fig. 2, Table 1). One explanation for the suppression of the drop in plasma NEFA level is as follows. Plasma NEFA levels are affected by adipose tissue lipolysis, which is regulated by hormones such as insulin and catecholamines, and by dietary intake of nutrition such as glucose (*16–18*). One of the important metabolic actions of insulin is the inhibition of lipolysis in adipose tissue (*19, 20*). Insulin secretion from islet β-cells is stimulated by a rise in blood glucose level (*21*). Therefore, after eating, blood glucose levels increase stimulating insulin secretion, which in turn causes a decrease in plasma NEFA levels due to the antilipolytic action of insulin. When CAS extract was given with food, the normal rise in blood glucose level was suppressed, and insulin secretion was also suppressed. Therefore, lipolysis in adipose tissue was not suppressed and plasma NEFA levels remained high.

α-Amylase inhibitors have been used in human health to inhibit excess energy supply, to control blood glucose levels, and to prevent or treat obesity and diabetes. A significant body of research has led to the discovery of many α-amylase inhibitors. Several have been reported in various plants, especially grains (*22, 23*), legumes (*24, 25*) and root vegetables (*26, 27*). In the present study, we discovered that a CAS extract was a strong inhibitor of α-amylase and could attenuate the rapid increase in blood glucose following consumption of a carbohydrate-containing meal by delaying or blocking absorption of carbohydrates. Chestnuts have been eaten since ancient times and CAS has also been eaten as an associated material. As an example, "Shibukawani" is a processed food, made by boiling the CAS-associated material with sugar. CAS is generated in large quantities as a waste product during CAS-free pea processing of chestnuts. Therefore, the use of CAS extract as a safe and inexpensive functional food or therapeutic agent adds potential value to chestnut processing.

In conclusion, this investigation suggests that CAS extract might exert an anti-diabetic effect by inhibiting α-amylase, and suppressing carbohydrate absorption from the intestine, thereby reducing post-prandial increase in blood glucose. Therefore, CAS extract may be useful as a potential additive to foods and beverages to inhibit carbohydrate adsorption.

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