Glycative Stress Research

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Original article

The effects of water chestnut (*Trapa bispinosa* Roxb.) on the inhibition of glycometabolism and the improvement in postprandial blood glucose levels in humans

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Abstract

***Objective*:** In the search for functional foods to reduce glycation stress, we evaluated the effects of the water chestnut peel extract (TBE) on glycometabolism-related enzymes α-amylase and α-glucosidase. Moreover, we assessed the impact of the extract on postprandial blood glucose levels in a human ingestion test and examined the possibility of using the extract as an anti-glycation stress food.

***Method*:** TBE's inhibitory effect on glycometabolism-related enzymes was evaluated as follows: in the presence of the test extract, we caused a reaction in each of the two pairs – α-amylase and starch, α-glucosidase and disaccharides – then evaluated the measured amount of resulting glucose using an *in vitro* test system. In addition, we tested 7 Japanese, healthy male and female subjects (3 male, 4 female, average age 23.1 ± 1.1 years) and 4 Japanese subjects (1 male, 3 female, average age 28.7 ± 2.3 years). The subjects ingested bread or rice after TBE intake, and we evaluated the subsequent swing in blood glucose levels to assess the impact of TBE on postprandial blood glucose levels on a comparative basis.

***Results*:** TBE showed concentration-dependent inhibitory activity on α-amylase and α-glucosidase. In humans, if the test extract was ingested before meals, the elevation in blood glucose levels 45, 60, and 90 minutes after bread intake and 15 minutes after rice intake was significantly lower ($p < 0.05$) than when the test extract was not ingested. In addition, the blood glucose area under the curve, AUC, was significantly lower (bread: $p = 0.012$, rice: $p < 0.01$) in the test extract group for both high-glycemic index foods.

***Conclusion*:** TBE showed an inhibitory effect on α-amylase and α-glucosidase. Further, trials of TBE ingestion in humans suggested its ability to suppress elevation of postprandial blood glucose levels. TBE is expected to become a functional food item that can have composite effects in countering glycation stress by controlling blood glucose levels.

***KEY WORDS*:** postprandial blood glucose levels, glycative stress, water chestnut (*Trapa bispinosa*)

Introduction

In diabetic patients with persistently high blood glucose levels, glycation reactions are accelerated compared to healthy subjects, causing early onset of diabetic nephropathy and eye disease [1], arteriosclerosis [2], osteoporosis [3], Alzheimer's disease [4], infertility [5], and skin hardening [6]. In addition, even in healthy subjects, an excessive increase in postprandial blood glucose levels and insulin secretion promotes the accumulation of visceral fat, elevating risks of lifestyle diseases such as dyslipidemia, hypertension, and arteriosclerosis [7, 8]. Therefore, proper management of blood glucose levels is important in reducing glycation stress and diabetes-related complications, as well as lifestyle diseases. In addition, it is believed that the suppression of glycation reactions (as part of glycation stress management) and the promotion of the decomposition and metabolism of existing saccharides will not only prevent diseases, but will also prolong life expectancy and improve quality of life (QOL). In light of this, the authors of this paper discovered strong anti-glycation and saccharide decomposition effects of water chestnut (*Trapa bispinosa* Roxb.) peel extract, and have researched and reported the possibilities around glycation stress reduction [9]. As part of the main experiment, this paper studies the inhibitory effect of the water chestnut hot water extract on glycometabolism-

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related enzymes and its ability to control blood glucose levels when ingested by humans.

Materials and Methods

Preparation of Water chestnut (T.bispinosa) Peel Extract (TBE)

Water chestnut peel was dried, sterilized, and crushed, and then extracted using hot water (approximately six-fold the weight of the water chestnut peel). Dextrin was added to this solution at a ratio of 67 : 33 of dry weight, and the resulting solution was spray-dried to obtain TBE. Sample solutions for subsequent measurements were prepared by dissolving the TBE in distilled water.

Measurement of α-glucosidase and α-amylase inhibition

TBE's inhibitory activity on α-glucosidase was measured according to the standards of prior research [10], and evaluated by measuring the amount of glucose produced after disaccharide decomposition. The α-glucosidase enzyme solution was obtained by dissolving intestinal acetone powder from rat (Sigma-Aldrich, Missouri, USA) in 0.1 mol/L-phosphate buffer solution (pH 7.0), then applying ultrasonic treatment while cooling on ice, and finally fractionating the supernatant by applying centrifugation at 800 x g over 10 minutes. We mixed 500 μL of TBE solution, each concentration of which was dissolved in distilled water, 2 mL of 250 mmol/L maltose or sucrose solution, and 2.4 mL of distilled water in a test tube, then pre-incubated at 37 °C for 5 minutes, before adding 100 μL of the above-mentioned α-glucosidase enzyme solution and causing a reaction during a 37 °C incubation for 40 minutes. After the reaction, 5 mL of 0.2 mol/L sodium carbonate was added to stop the reaction and a measurement sample was removed. The Glucose CII-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) kit was used to determine the amount of glucose contained in the sample in accordance with the manufacturer's instructions. A mixture of 3 mL of the color former included in the kit and 0.02 mL of the measurement sample was incubated at 37 °C for 5 minutes, and the absorbance was measured at 505 nm using a microplate reader (Tecan, Salzburg, Austria). The inhibitory activity of TBE on glucosidase was then calculated using the following equation. The treatment group described above is [S], the control test group [C] used distilled 0.02 mL of water instead of TBE, and a third test group [SB] in which the solution with the halted chemical reaction was added in the presence of TBE and then mixed with enzyme solution. For each test case we obtained the value of absorbance and calculated the inhibitory activity of TBE on the α-glucosidase enzyme using the following equation.

$$\alpha\text{-glucosidase inhibitory activity (\%)} = 100 - 100 \times ([S] - [SB])/[C]$$

We evaluated the inhibitory activity of TBE on α-amylase using a positive control amount of glucose produced by enzymatic degradation of soluble starch, as cited in previous research [11]. In a microtube, 0.4% soluble starch was added to 50 μL TBE solution dissolved in distilled water and 300 μL of 25 mmol/L PIPES buffer containing 5 mmol/L calcium chloride (pH 6.0), and mixed thoroughly to create a reaction composition solution. To this reaction composition solution we added 150 μL of enzyme solution, obtained by dissolving porcine pancreatic α-amylase (Sigma-Aldrich, Missouri, USA) in 25 mmol/L PIPES buffer solution to form 2 units/mL. The reaction was carried out over a 10 minute incubation period at 37 °C. The enzymatic reaction was stopped by heating in boiling water for 10 minutes. After cooling, 100 μL of 2 mol/L sodium hydroxide aqueous solution and 200 μL of 0.5% dinitrosalicylic acid were added to the reacted solution and heated in boiling water for 10 minutes. After cooling, the absorbance was measured at 540 nm using a microplate reader. We labeled the test group described above as [S], a control test group [C] in which distilled water was added instead of TBE, a third test group [B] in which distilled water was added instead of TBE and enzyme solution, and the final test group [SB] in which distilled water was added instead of the enzyme solution. For each test case we obtained the value of absorbance and calculated the inhibitory activity of TBE on the α-amylase enzyme using the following equation.

$$\alpha\text{-amylase inhibitory activity (\%)} = 100 - 100 \times ([S] - [C]/([B] - [C])$$

As a comparison, in place of TBE, we simultaneously carried out tests using acarbose, an oral hypoglycemic drug with an inhibitory effect on α-amylase and α-glucosidase, and the leaf extract of mulberry (*Morus alba* Linn.) which is reported to have similar inhibitory effects [12] on glycometabolism-related enzymes.

Human Clinical Study

Subjects

The subjects were selected from healthy Japanese men and women of ages 20 years or older, for whom the following exclusion criteria did not apply:

Exclusion criteria: patients with diabetes, anyone taking medication, smokers, anyone with food or drug allergies, women who are pregnant or nursing, anyone with a past history of severe symptoms or surgery of the digestive tract, anyone taking daily supplements.

7 subjects were tested with bread (3 males, 4 females, average age 23.1 ± 1.1 years of age), and 4 subjects were tested with rice (1 male, 3 females, average age 29.0 ± 2.4 years).

Study Design

Referencing the methodology of prior research [13], we tested as outlined below. The subjects finished dinner by 10 pm on the night before the experiment, skipped breakfast, and drank only water until the test. In addition, the subjects abstained from alcohol the day before the test.

Measurements of blood glucose levels were carried out in the following manner. The subject's fingertip was disinfected with rubbing alcohol and then a lancing device was used to test a small amount of blood using a blood glucose meter (Medisafe Mini by Terumo, Tokyo, Japan or Glucocard MyDia by Arkray, Kyoto, Japan). Two blood glucose levels were simultaneously measured and the averaged value was used. If the difference between the two measurements was more than 10% of the higher value, a

third measurement was taken and the average value of two measurements with a smaller difference was used.

During the test the subject's fasting blood sugar level was measured, subjects were administered the test extract with 100 mL water, and the elimination food was ingested 5 minutes later. The ingestion time of the elimination food was 10 minutes, and the rate of ingestion was kept constant. After ingesting the elimination food we measured blood glucose levels after 15 minutes, 30 minutes, 45 minutes, 60 minutes, 90 minutes, and 120 minutes. Collected data was used to evaluate the blood glucose levels, fluctuations in blood glucose levels, and the blood glucose area under the curve (AUC). With regard to the elimination food, the tests were carried out separately for bread and rice, and we performed a comparison of the blood glucose levels when ingesting only the elimination food and when ingesting the tes t extract and elimination food. In consideration of the lingering effects of the ingested test extract, each test was carried out at 1 week intervals.

The Characteristics of the Test Extract and Elimination Food

The ingested test extract and elimination food is outlined below. ***Table 1*** & ***2*** show their composition and nutritional components.

Test extract: gelatin hard capsules containing 100 mg of TBE

Elimination Food 1: 170 g bread (Pasco Chojuku) (Shikishima Corporation, Aichi, Japan)

Elimination Food 2: 200 g packaged rice (TableMark Co., Ltd., Tokyo, Japan) + 2.5 g seasoning (Nagatanien Co. Ltd., Tokyo, Japan)

Ethical considerations

This study complies with the ethical principles and the protection of personal information based on the Declaration of Helsinki (annotations added at the 2004 Tokyo meeting). The experiment was carefully explained to the subjects in writing, and consent to participate in the experiment was also obtained in writing. We also held an ethics committee regarding Doshisha University's ethical standards, "Experimentation Involving Human Subjects," to deliberate the ethics and validity of the test. The experiment was approved (Application Number: 1228-2).

Statistical Analysis

Test results are expressed as mean ± standard deviation. A Student's t-test was used to compare the test and control groups. We set the significance level of the two-sided test to 5% and considered values less than or equal to this to be statistically significant.

Results

Measurement of α-glucosidase and α-amylase inhibition

Figure 1 shows a graph that depicting the α-glucosidase inhibitory activity of TBE, while the graph in ***Fig. 2*** shows the α-amylase inhibitory activity. Data in ***Fig. 1*** confirms the dose-dependent increase in the α-glucosidase (maltase, sucrase) inhibitory activity of TBE. The IC_{50} value was 571 μg/mL for maltase and 548 μg/mL for sucrase. The IC_{50}

Table 1. Nutritional facts of the standard diet.

	Serving weight (g)	Carbohydrate (g)	Energy (kcal)	Protein (g)	Fat (g)
(A) Bread					
Bread	170	79.4	448.8	15.8	7.5
(B) Rice and seasoning					
Rice	200	69	299	4.4	0.6
Seasoning	2.5	1.2	9	0.7	0.13

Table 2. Composition and nutritional factor of the test diet.

Composition per serving		**Nutritional factor per serving**	
TBE (mg)	100	Carbohydrate (g)	0.25
Cornstarch (mg)	187	Energy (kcal)	1
Calcium stearate (mg)	3	Protein (g)	0
Total (mg)	290	Fat (g)	0

Test diet is served as a capsule which contains 100 mg TBE. TBE, water chestnut peel extract.

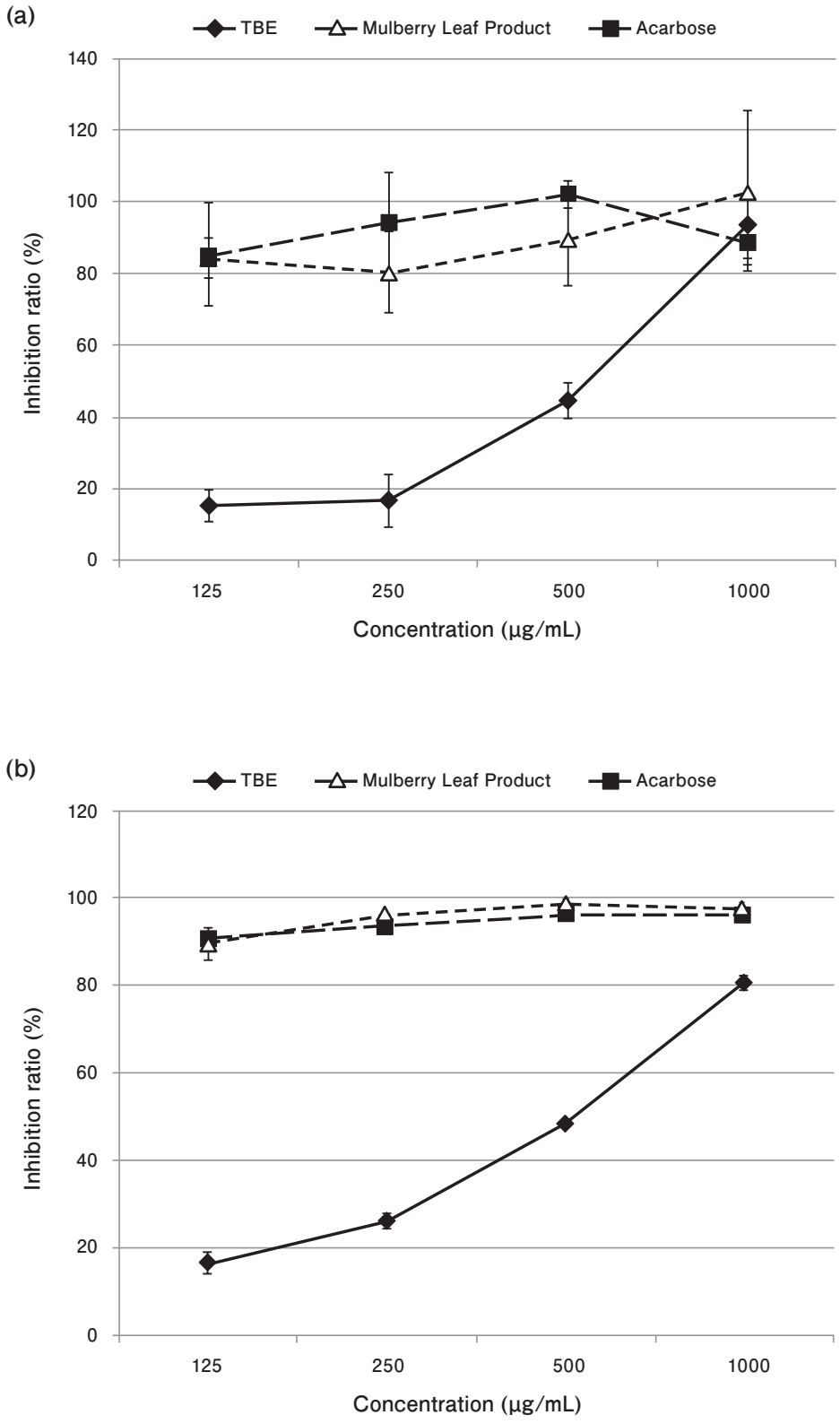

Fig. 1. **Inhibitory activitiy of TBE, mulberry leaf product and acarbose on (a) sucrase, (b) maltase.**
TBE, water chestnut peel extract.

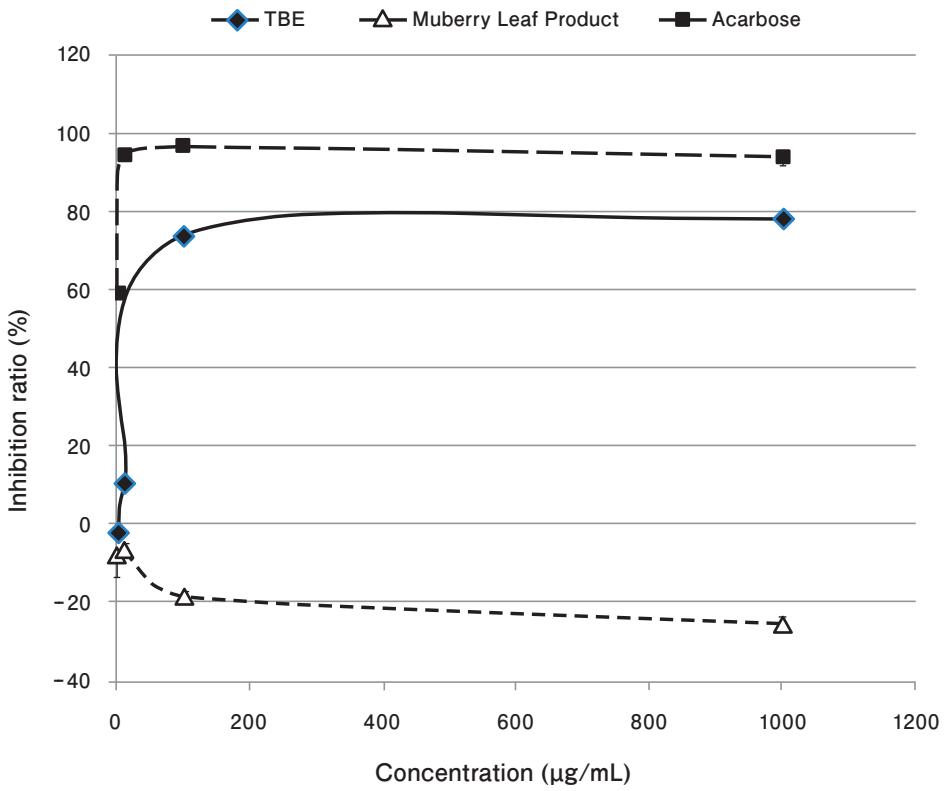

Fig. 2. **Inhibitory activity of TBE, mulberry leaf product and acarbose on α-amylase.**
TBE, water chestnut peel extract.

values of both acarbose and mulberry leaf extract, used as comparative controls, were <125 mg/mL, with confirmed high activity in comparison with TBE. Similarly, data in ***Fig. 2*** confirmed the dose-dependent increase of α-amylase inhibitory activity of TBE; the IC_{50} value was 67.3 μg/mL. The IC_{50} value of acarbose, which had been used as a comparative control, was found to be <1 μg/mL, having a higher activity in comparison with TBE. On the other hand, α-amylase inhibitory activity was not observed in the mulberry leaf extract.

Human Clinical Study

Table 3 shows blood glucose levels after the ingestion of each elimination food, the amount of change in blood glucose levels, and the AUC value. ***Figure 3*** depicts a graph of the

Table 3. Effects of TBE on blood glucose and area under the glucose curve (AUC) in human.

		Time after intake (minutes)						
	Group	**0**	**15**	**30**	**45**	**60**	**90**	**120**
Blood glucose level	**B**	79.8 ± 7.5	92.9 ± 15.0	131.1 ± 14.5	144.6 ± 20.8	142.9 ± 31.7	124.8 ± 31.7	118.9 ± 30.7
	B + TBE	93.8 ± 5.7	100.0 ± 10.0	144.1 ± 20.2	139.6 ± 12.5	128.6 ± 15.7	113.4 ± 24.8	110.6 ± 19.5
	R	84.1 ± 4.3	133.0 ± 4.1	137.0 ± 9.4	115.4 ± 5.5	102.1 ± 4.6	104.6 ± 2.7	91.8 ± 5.9
	R + TBE	85.6 ± 2.5	114.9 ± 2.9	128.3 ± 7.4	114.8 ± 7.7	99.4 ± 8.0	90.8 ± 6.7	91.5 ± 3.1
ΔBlood glucose level	**B**		13.1 ± 5.6	51.3 ± 5.1	64.8 ± 6.8	63.1 ± 11.2	45.0 ± 11.0	39.1 ± 10.5
	B + TBE		6.2 ± 4.3	50.3 ± 8.6	45.8 ± 5.8*	37.8 ± 6.4*	19.6 ± 8.6*	16.9 ± 7.2
	R		48.9 ± 4.1	52.9 ± 8.3	31.3 ± 2.9	18.0 ± 5.6	20.5 ± 2.4	7.6 ± 2.1
	R + TBE		29.3 ± 4.8*	42.6 ± 7.1	29.1 ± 6.2	13.8 ± 7.1	5.1 ± 7.1	5.9 ± 3.0
		AUC						
Area under the glucose curve (AUC)	**B**	5298.2 ± 779.4						
	B + TBE	3236.2 ± 570.0*						
	R	3129.2 ± 623.4						
	R + TBE	2167.5 ± 551.5*						

Intook bread (n = 7); group B, intook bread after TBE; group B + TBE. Intook rice (n = 4); group R, intook rice after TBE; group R + TBE. Results are expressed as mean ± standard deviation. * $p < 0.05$, group B *vs* group B + TBE and group R *vs* group R + TBE, by Student's t-test. TBE, water chestnut peel extract.

amount of change in blood glucose levels. ***Figure 4*** shows a graph charting AUC. From ***Table 3*** and ***Fig. 3***, significantly lower blood glucose levels ($p < 0.05$) were observed in the test group that ingested TBE before bread intake (Group B + TBE) compared to the test group with just bread intake (Group B) at 45, 60, and 90 minutes after ingestion. In addition, significantly lower blood glucose levels ($p < 0.05$) were observed for the test group that ingested TBE before rice intake (Group R + TBE) in comparison to the group with just rice intake (Group R) 15 minutes after ingestion. From ***Fig. 4***, the AUC of Group B + TBE (3,236.2 ± 570) is significantly lower ($p = 0.012$) compared to the AUC of Group B (5,298.2 ± 779.4). Likewise, the AUC of Group R + TBE (2,167.5 ± 551.5) was significantly lower ($p < 0.01$) compared to the AUC of Group R (3,129.3 ± 623.4).

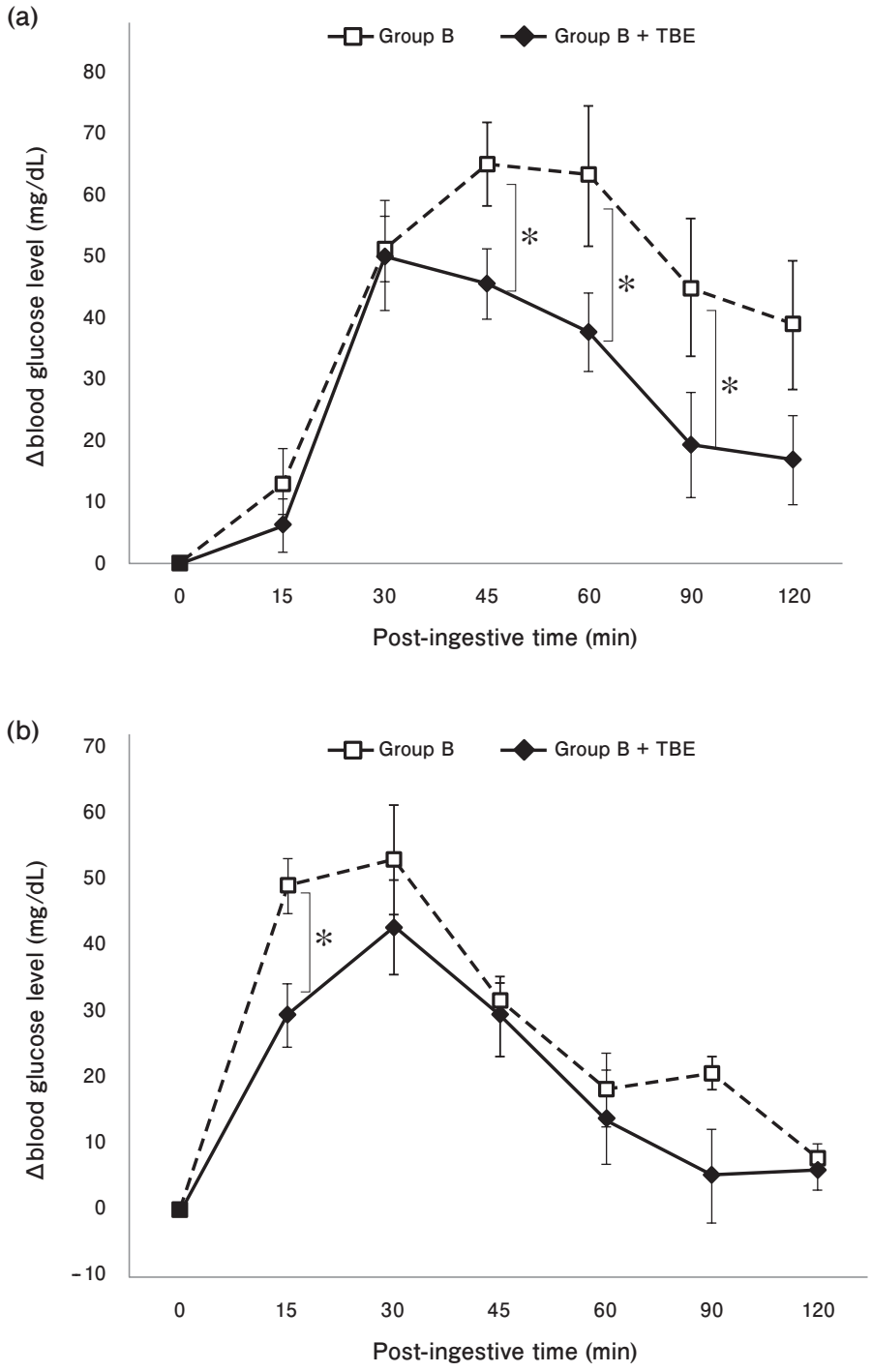

Fig. 3. **Effects of TBE on postprandial blood glucose level on subjects in (a) bread or (b) rice.**

Test subjects were loaded (a) bread (n = 7) or (b) rice (n = 4). (a) Intook bread; group B, intook bread after TBE; group B + TBE. (b) Intook rice; group R, intook rice after TBE; group R + TBE. Results are expressed as mean ± standard deviation. * $p<0.05$, group B *vs* group B + TBE and group R *vs* group R + TBE, by Student's t-test. TBE, water chestnut peel extract.

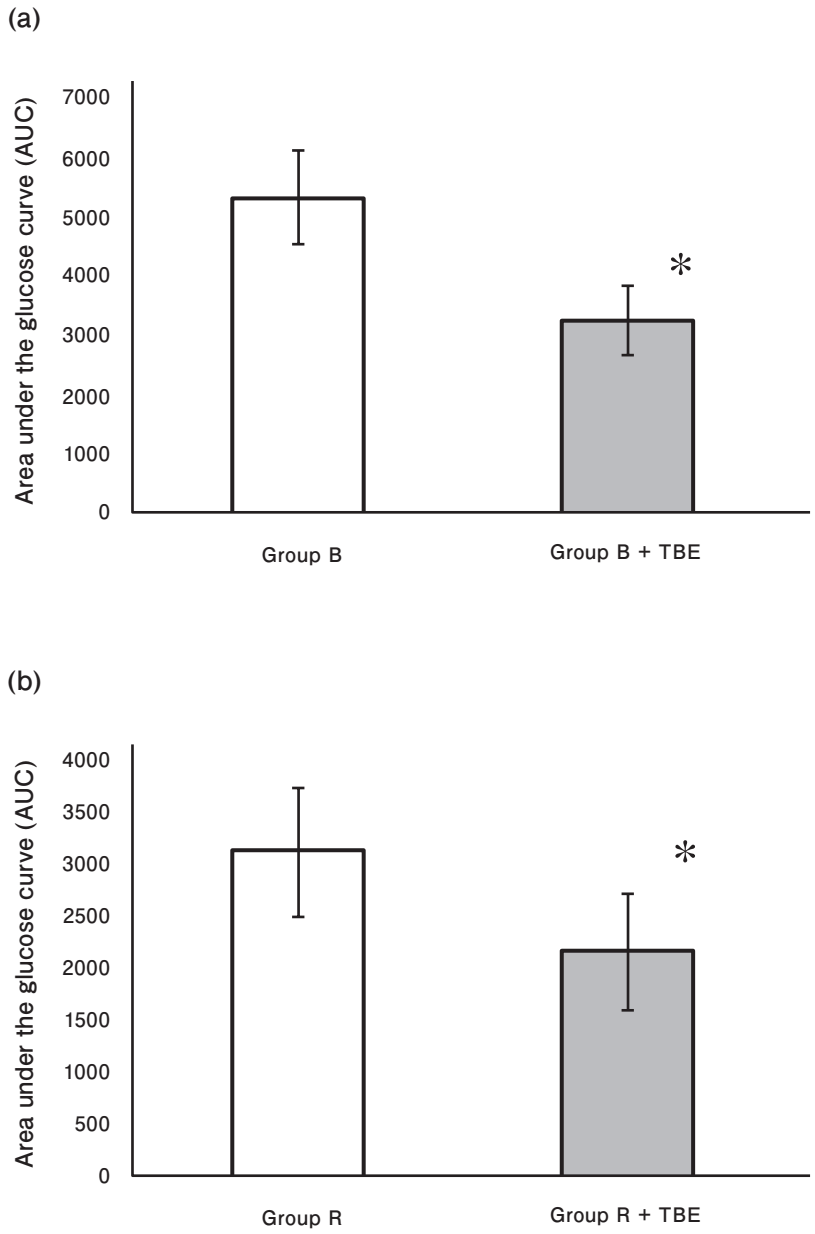

Fig. 4. **Effects of TBE on area under the glucose curve (AUC) in (a) bread or (b) rice.**
Test subjects were loaded (a) bread (n = 7) or (b) rice (n = 4). (a) Intook bread; group B, intook bread after TBE; group B + TBE. (b) Intook rice; group R, intook rice after TBE; group R + TBE. Results are expressed as mean ± standard deviation. * $p < 0.05$, group B *vs* group B + TBE and group R *vs* group R + TBE, by Student's t-test. TBE, water chestnut peel extract.

Discussion

The water chestnut fruit used as the subject of this study is a large fruit among plants in the Trapacese family [14]. In Japan, water chestnut has been in circulation since ancient times in the Fukuoka and Saga prefectures, where the peel is dried and used as a specialty tea. However, the benefits of water chestnut intake are limited to oral tradition and folklore, and there are few research reports. Therefore, to thoroughly study the subject of glycation slowdown with respect to glycemic control, we focused on TBE's strong anti-glycation activity and evaluated its effects on glycometabolism-related enzymes, and its impact on postprandial blood glucose levels in humans.

TBE's inhibitory activity was examined on the glycometabolism-related enzymes α-glucosidase and α-amylase, both of which regulate blood glucose levels. We confirmed TBE to inhibit enzymatic activity in a dose-dependent manner. When compared with the mulberry leaf extract, a positive control group, the inhibitory activity of TBE on α-glucosidase was weaker than the mulberry leaf extract, but its inhibitory effect on α-amylase was higher than the mulberry leaf extract.

The active ingredient in the α-glucosidase inhibitory activity of the mulberry leaf extract has been found to be 1-deoxynojirimycin, which is an analog of glucose. Indeed, high inhibitory activity is reported when 1-deoxynojirimycin binds specifically to α-glucosidase [12]. On the other hand, it has been reported that the mulberry leaf extract is poor in α-amylase inhibitory activity, which is consistent with the results of this study [15]. Previous reports show that the pericarp extract of plants in the Trapa genus contain polyphenols [16], and inhibitory activity on glycometabolism-related enzymes has been confirmed in polyphenols such as eugeniin and trapain contained in the pericarp extract of the *Trapa japonica* [11]. Thus the inhibitory activity of TBE on glycometabolism-related enzymes has been partially attributed to polyphenols, and it is believed to be a different mechanism than the mulberry leaf extract. α-glucosidase (maltase, sucrase) is a disaccharide-degrading enzyme located in the intestinal tract, and α-amylase is a starch-degrading enzyme found in saliva and pancreatic fluid. It is thought that, because TBE inhibits the activity of both glycometabolism-related enzymes, it is able to suppress the degradation of starch, sucrose, and maltose into glucose.

In the human clinical trials, we evaluated blood glucose fluctuations after ingesting TBE and elimination foods. Bread and rice, which were used as elimination foods, have glycemic indices (GI values) of 70 or more. The GI values represent an index of the swing in postprandial blood glucose levels proposed by Jenkins DJ *et al.* [17]. Bread and rice cause a rapid rise in blood glucose levels, and are commonly modeled to observe the swing in postprandial blood glucose levels. The results of the TBE ingestion test show that the ingestion of TBE before the elimination food caused the swing in blood glucose levels to be significantly lower in comparison to the test group that did not consume TBE, at 45, 60, and 90 minutes after bread consumption, and 15 minutes after rice consumption. For both elimination foods, the maximum blood sugar value was lower with TBE intake than without, and this is inferred to be due to TBE's inhibitory activity on glycometabolism-related enzymes as shown in the *in vitro* test described above. The AUC value, the indicator of total change in blood glucose level, was also significantly lower during TBE intake in comparison to no intake. The difference in the AUC values of bread and rice is thought to be attributable to the differences in GI value and carbohydrate content.

This study suggests that the ingestion of TBE eases the rise of postprandial blood glucose levels. Its mechanism is thought to be a result of delayed absorption or suppression of glucose in the intestines due to TBE's inhibitory activity on glycometabolism-related enzymes, α-glucosidase and α-amylase. With regards to TBE's ability to reduce glycation stress, we have already discovered and reported on its high inhibitory effect on glycation and saccharide decomposition [9]. It is presumed that our study on TBE's potential to control postprandial blood glucose levels may further contribute to the reduction of glycation stress.

This study helped to gain knowledge on the impact of a single ingestion of TBE on the control of postprandial blood glucose levels. Since the long-term ingestion of TBE is believed to lead to suppression of *in vivo* formation of AGEs, we look to further continue our research.

Conclusion

TBE showed an inhibitory effect on α-amylase and α-glucosidase. Further, trials of TBE ingestion in humans suggested its ability to suppress elevation of postprandial blood glucose levels. TBE is expected to become a functional food item that can have composite effects in countering glycation stress by controlling blood glucose levels.

Conflicts of interest statement

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