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RESEARCH ARTICLE

A series of cinnamic acid derivatives and their inhibitory activity on intestinal α-glucosidase

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Abstract
Inhibition of α-glucosidase and α-amylase delays the digestion of starch and disaccharides to absorbable monosaccharides, resulting in a reduction of postprandial hyperglycemia. Finding effective mammalian α-glucosidase inhibitors from natural sources can be beneficial in the prevention and treatment of diabetes mellitus. We investigated the inhibitory activity of cinnamic acid derivatives against rat intestinal α-glucosidase and porcine pancreatic α-amylase *in vitro*. Among 11 cinnamic acid derivatives, caffeic acid, ferulic acid, and isoferulic acid were the most potent inhibitors against intestinal maltase with IC_{50} values of 0.74 ± 0.01, 0.79 ± 0.04, and 0.76 ± 0.03 mM, respectively, whereas ferulic acid ($IC_{50} = 0.45 \pm 0.01$ mM) and isoferulic acid ($IC_{50} = 0.45 \pm 0.01$ mM) were effective intestinal sucrase inhibitors. However, all cinnamic acid derivatives were found to be inactive in pancreatic α-amylase inhibition. Kinetic analysis revealed that intestinal maltase was inhibited by caffeic acid, ferulic acid, and isoferulic acid in a mixed-inhibition manner. In addition, ferulic acid and isoferulic acid inhibited intestinal sucrase in a mixed type manner, whereas caffeic acid was a non-competitive inhibitor. The combination of isoferulic acid and acarbose showed an additive inhibition on intestinal sucrase. This study could provide a new insight into naturally occurring intestinal α-glucosidase inhibitors that could be useful for treatment of diabetes and its complications.

Keywords: *Cinnamic acid derivatives; intestinal α-glucosidase; pancreatic α-amylase; enzyme kinetics; inhibition*

Introduction

Diabetes mellitus is in the group of metabolic diseases characterized by hyperglycemia, dyslipidemia, and protein metabolism that result from defects in both insulin secretion and/or insulin action[1]. The disease is associated with a reduced quality of life and increased risk factors for mortality and morbidity. Inhibition of α-glucosidase and pancreatic α-amylase is one of the therapeutic approaches for delaying oligosaccharide digestion to absorbable monosaccharides, resulting in reduced postprandial hyperglycemia[2]. Inhibitors of these enzymes also have a potential to reduce the progression of diabetes, as well as micro- and macrovascular complications such as retinopathy, nephropathy, diabetic neuropathy, and sexual dysfunction[3].

An intake of plant foods and their ingredients could be a more effective strategy for the management of diabetes mellitus, because this is largely free from side effects. Many studies have focused on the potential biological activities or health effects of phytochemical compounds in animals and humans. In particular, cinnamic acid and its derivatives refer to one of the numerous and widely distributed groups of molecules in the plant kingdom. Interestingly, the focus has been on cinnamic acid derivatives regarding their potential benefits in antioxidative[4], hepatoprotective[5], and antityrosinase related[6] as well as antidiabetic activities[7].

For example, ferulic acid (3-methoxy-4-hydroxycinnamic acid) is an effective scavenger of free radicals, prevents lipid peroxidation, and effectively suppresses the blood glucose level in diabetic mice[8]. Furthermore, isoferulic acid significantly decreases the levels of plasma glucose concentration in streptozotocin (STZ)-induced diabetic rats by suppression of hepatic gluconeogenesis[7]. Our previous report has demonstrated the inhibitory activities of cinnamic acid derivatives against yeast α-glucosidase[9]. Currently, yeast α-glucosidase is known to be structurally different from those of mammalian origin. Accordingly, the study of α-glucosidase inhibition from mammalian sources may be a better model in the design and development of antihyperglycemic agents[10].

Therefore, it would be interesting to investigate the structure–activity relationships of cinnamic acid and its derivatives with intestinal α-glucosidase (maltase and sucrase) and pancreatic α-amylase inhibition. The present work aimed to carry out a kinetic study of the inhibition of intestinal α-glucosidase by cinnamic acid derivatives in order to evaluate the types of inhibition. Finally, the combined effects of cinnamic acid derivatives and acarbose on α-glucosidase inhibition were investigated.

Materials and methods

Reagents

Rat intestinal acetone powder, porcine pancreatic α-amylase, and glucose oxidase kits were from Sigma-Aldrich (St. Louis, MO, USA). Acarbose was obtained from Bayer (Germany). Cinnamic acid, *o*-hydroxycinnamic acid, *m*-hydroxycinnamic acid, caffeic acid (3,4-dihydroxycinnamic acid), 3,4-dimethoxycinnamic acid, and *p*-hydroxycinnamic acid were from Fluka (St. Louis, MO, USA.). *o*-Methoxycinnamic acid, *m*-methoxycinnamic acid, and *p*-methoxycinnamic acid were obtained from ACROS (Pittsburgh, PA, USA). Ferulic acid (3-methoxy-4-hydroxycinnamic acid) and isoferulic acid (4-methoxy-3-hydroxycinnamic acid) were from Chromadex (Laguna Hills, CA, USA). All other chemicals used were of analytical grade.

Assay for intestinal α-glucosidase inhibitory activity

The assessment of intestinal α-glucosidase inhibitory activity was slightly modified, according to a previous method[11]. Briefly, 100 mg of rat intestinal acetone powder was homogenized in 3 mL of 0.9% NaCl solution. After centrifugation (12,000 $g \times$ 30 min), the solution was subjected to assay, and the specific activities of sucrase and maltase were 0.20 and 0.68 units/mg protein, respectively. The crude enzyme solution (as maltase assay, 10 μL; as sucrase assay, 40 μL) was incubated with 70 μL maltose or 40 μL sucrose (final concentration: 25.9 mM maltose; 48 mM sucrose) in 0.1 M phosphate buffer, pH 6.9, and 20 μL of test compound at various concentrations in dimethyl sulfoxide (DMSO) at 37°C for 30 min (maltase assay) or 60 min (sucrase assay). The mixtures were suspended in boiling water for 10 min to stop the reaction. The concentrations of glucose released from the reaction mixtures were determined by the glucose oxidase method.

Assay for porcine α-amylase inhibitory activity

The pancreatic α-amylase inhibition assay was slightly modified, according to the literature procedure[12]. Porcine pancreatic α-amylase was dissolved in 0.1 M phosphate buffered saline, pH 6.9. Various concentrations of test compound were added to a solution containing 1 g/L starch and phosphate buffer. The reaction was initiated by adding amylase (3 U/mL) to the incubation medium to a final volume of 500 μL. After 10 min the reaction was stopped by adding 0.5 mL dinitrosalicylic (DNS) reagent (1% 3,5-dinitrosalicylic acid, 0.2% phenol, 0.05% Na_2SO_3, and 1% NaOH in aqueous solution) to the reaction mixture. Mixtures were heated at 100°C for 10 min to develop a yellow-brown color. Thereafter, 500 μL of 40% potassium sodium tartrate solution was added to the mixture to stabilize the color. After cooling the mixtures to room temperature in a cold water bath, absorbance was recorded at 540 nm using a spectrophotometer.

Measurement of kinetic constant

In order to investigate the type of inhibition, enzyme kinetic analysis was performed according to the above reaction. Maintaining the quantity of sucrase constant at 0.20 units/mg protein or 0.68 units/mg protein, test compounds (0 and 0.25 mM) were measured for final concentrations of maltose (6.5, 12.8, 25.9, 51.5 mM) and sucrose (21.6, 32, 48, 64.8 mM). The type of inhibition was calculated on the basis of a Lineweaver–Burk plot using reciprocally plotted data (substrate concentration on horizontal axis and velocity on vertical axis)[13].

Combined effect of cinnamic acid derivatives and acarbose

Acarbose (1 μM) was combined with or used without the cinnamic acid derivatives (0.031 mM). The reaction was performed according to the above assay. Results are expressed as percentage inhibition compared to the corresponding control values.

Data analysis

Data are expressed as mean ± SEM ($n = 3$). IC_{50} values were determined from plots of log concentration of inhibitor versus percentage inhibition. The type of inhibition and constants were calculated on the basis of Lineweaver–Burk plots and non-linear regression of the replot of slopes using Sigma Plot 10.0 software (IL, USA). Statistical analysis was performed by Student's *t*-test ($p < 0.01$).

Results

Screening of cinnamic acid derivatives as intestinal α-glucosidase and porcine α-amylase inhibitors

Eleven compounds of cinnamic acid and its derivatives (Figure 1) were investigated for intestinal α-glucosidase (maltase and sucrase) and α-amylase inhibition. The results in Figure 2 show the percentage enzyme inhibition of test agents at a concentration of 2 mM. Cinnamic acid **(1)**
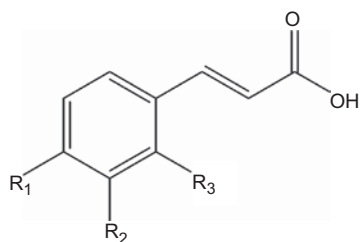
Compound	R₁	R₂	R₃	Chemical name
1	H	H	H	Cinnamic acid
2	H	H	OH	o-Hydroxycinnamic acid
3	H	OH	H	m-Hydroxycinnamic acid
4	OH	H	H	p-Hydroxycinnamic acid
5	H	H	OCH₃	o-Methoxycinnamic acid
6	H	OCH₃	H	m-Methoxycinnamic acid
7	OCH₃	H	H	p-Methoxycinnamic acid
8	OH	OH	H	Caffeic acid
9	OCH₃	OCH₃	H	3,4-Dimethoxycinnamic acid
10	OH	OCH₃	H	Ferulic acid
11	OCH₃	OH	H	Isoferulic acid

Figure 1. Structure of cinnamic acid (**1**) and its cinnamic acid derivatives (**2–11**).
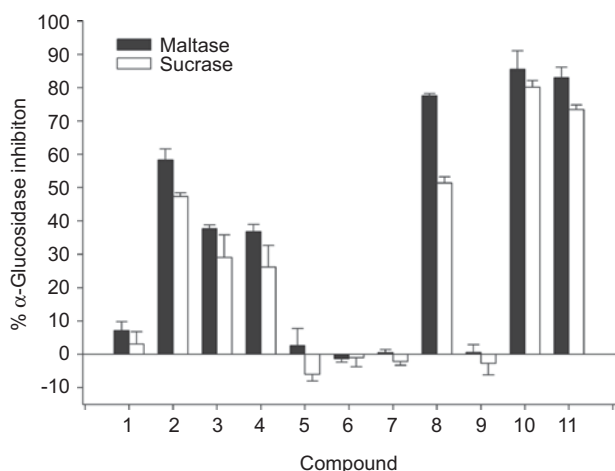

Figure 2. Percentage α-glucosidase inhibition of cinnamic acid and its derivatives. Data are expressed as mean ± SEM; $n = 3$.

showed a weak inhibitory activity against intestinal maltase and sucrase. Upon the introduction of a hydroxyl group to cinnamic acid at various positions, it was found that the intestinal maltase and sucrase inhibitory activities increased in the order of **2 > 3 = 4**. In addition, replacement of a hydroxyl residue in the cinnamic acid by a methoxyl residue (**5–7**) resulted in a significant loss of intestinal maltase and sucrase inhibitory activities. The addition of two hydroxyl substituents at *meta* and *para* positions in cinnamic acid

Table 1. IC_{50} values for intestinal α-glucosidase (maltase and sucrase) inhibition by cinnamic acid derivatives.

Compound	IC_{50} value (mM)	
	Maltase	Sucrase
8	0.74 ± 0.01	0.49 ± 0.01
10	0.79 ± 0.04	0.45 ± 0.01
11	0.76 ± 0.03	0.45 ± 0.01

Note. Results are expressed as mean ± SEM; $n = 3$.

(8) exhibited higher potency than that of the presence of two methoxyl substituents at the same positions **(9)**. Interestingly, replacement of a hydroxyl residue at the *meta* or *para* position of **8** by a methoxyl moiety (**10**, **11**) did not affect the inhibitory activity against intestinal maltase, but it increased the intestinal sucrase inhibitory activity. Likewise, all test compounds did not show any effect on α-amylase inhibition (data not shown).

Determination of IC_{50} values for intestinal maltase and sucrase activities

As mentioned above, **8**, **10**, and **11** were effective compounds against α-glucosidase inhibition. Thus, we further examined the IC_{50} values of these compounds against intestinal maltase and sucrase (Table 1). They exhibited selective inhibition of α-glucosidase toward sucrase rather than maltase. The IC_{50} values for intestinal maltase inhibition by **8**, **10**, and **11** were 0.74 ± 0.01, 0.79 ± 0.04, and 0.76 ± 0.03 mM, respectively. In addition, IC_{50} values for intestinal sucrase inhibition by **8**, **10**, and **11** were 0.49 ± 0.01, 0.45 ± 0.01, and 0.45 ± 0.01 mM, respectively. However, these compounds were less potent than acarbose on intestinal maltase ($IC_{50} = 2.2 ± 0.6\,\mu M$) and sucrase inhibition ($IC_{50} = 4.8 ± 1.2\,\mu M$).

Inhibition kinetic parameters of cinnamic acid derivatives on intestinal maltase and sucrase

To further explore the inhibitory characteristics of cinnamic acid derivatives, kinetic assay was performed using Lineweaver–Burk double reciprocal plots. The inhibition mechanisms on intestinal maltase by **8**, **10**, and **11** are shown in Figure 3. Lineweaver–Burk plots for **8**, **10**, and **11** generated straight lines which had different intersections on the x-axis in the second quadrant, indicating that their inhibitions were of the mixed competitive and non-competitive type.

The inhibition mechanisms on intestinal sucrase by **8**, **10**, and **11** are shown in Figure 4. Lineweaver–Burk plots for **10** and **11** generated straight lines which had a point of intersection in the second quadrant, suggesting that their inhibitions were of the mixed competitive and non-competitive type, whereas **8** generated straight lines which intersected on the x-axis, indicating that its type of inhibition was non-competitive.

Combined effect of cinnamic acid derivatives and acarbose

It was of interest to establish whether **8**, **10**, and **11** and acarbose might interact synergistically or additively on α-glucosidase. The assay was then performed using
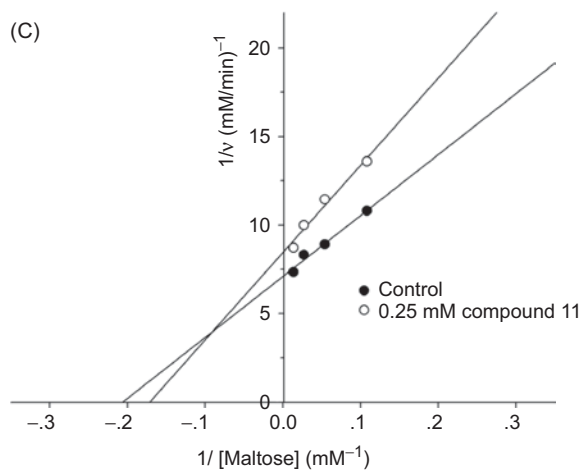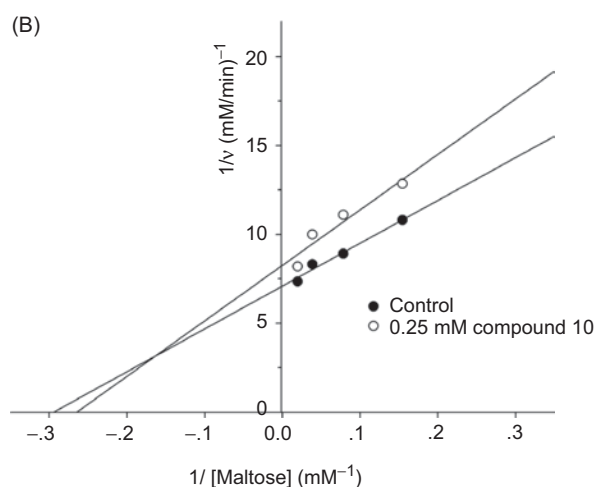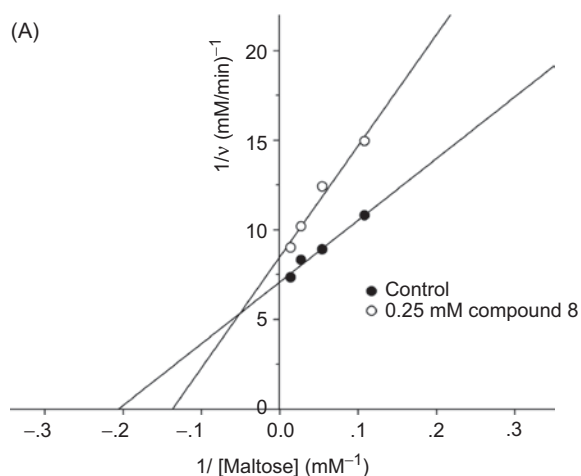
Figure 3. Lineweaver–Burk plots for inhibitory activity of (A) compound **8**, (B) compound **10**, and (C) compound **11** on intestinal maltase.

Figure 4. Lineweaver–Burk plots for inhibitory activity of (A) compound **8**, (B) compound **10**, and (C) compound **11** on intestinal sucrase.

solutions containing acarbose alone or mixtures with a low dose of these compounds (0.03 mM). The effects of **8**, **10**, and **11** with acarbose on intestinal maltase and sucrase inhibition are shown in Figure 5. Upon addition of **8**, **10**, and **11** to the assay system with acarbose (1 μM), the percentage sucrase inhibition was increased when compared with
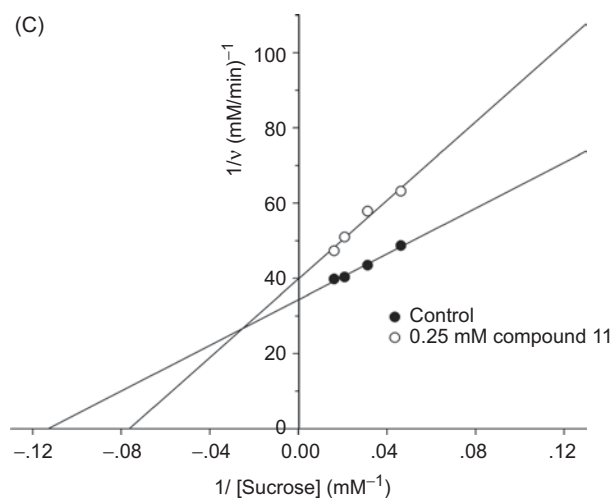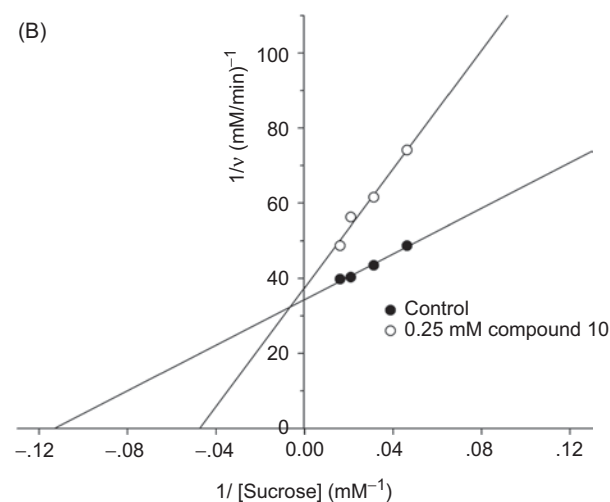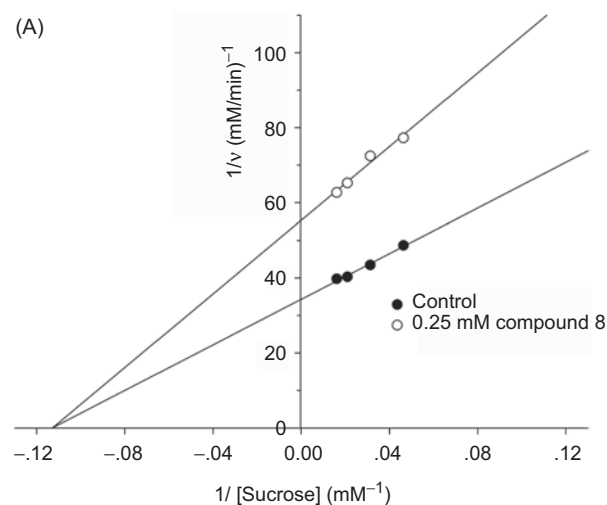
acarbose alone. The percentage inhibition using the mixtures was equal to the summing effect of acarbose and these compounds. These findings indicate that the combination of the compounds and acarbose produced additive inhibition
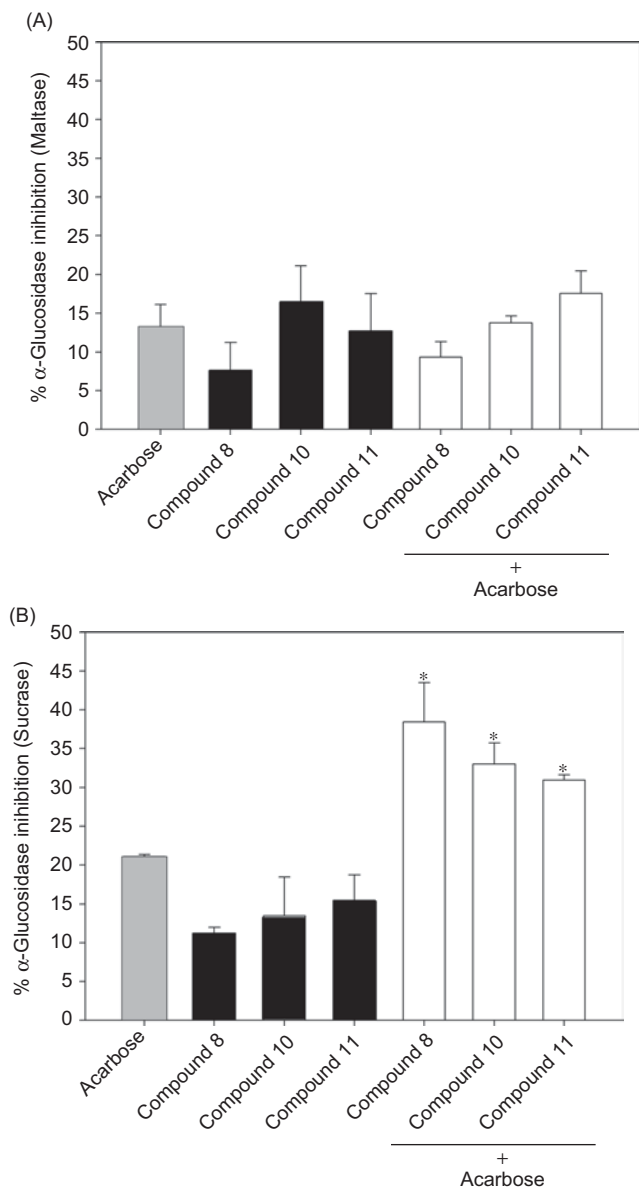
Figure 5. Percentage enzyme inhibition of acarbose and its combination with compounds **8**, **10**, and **11** on (A) intestinal maltase and (B) intestinal sucrase. Results are expressed as mean ± SEM; $n = 3$. *$p < 0.01$ compared to acarbose alone.

against intestinal sucrase. However, there was no increase in percentage maltase inhibition when these compounds were combined with acarbose, indicating that **8**, **10**, and **11** did not produce any additive or synergistic inhibition with acarbose in this case.

Discussion

This is the first study to investigate the structure–activity relationships of cinnamic acid and its derivatives with regard to intestinal α-glucosidase inhibition (maltase and sucrase). In the present study, it was found that cinnamic acid is a weak intestinal α-glucosidase inhibitor. Our findings indicate that the presence of one substituent by a hydroxyl moiety is important for inducing potent inhibition against intestinal

maltase and sucrase. Moreover, the findings also suggest that the presence of two hydroxyl groups at the *meta* and *para* positions of cinnamic acid can increase the potency of intestinal maltase inhibition. The conversion of one hydroxyl group at the *meta* or *para* position to a methoxyl group did not affect inhibitory activity on intestinal maltase; in contrast, it dramatically enhanced the potency of intestinal sucrase inhibition. The explanation for interaction phenomena between cinnamic acid derivatives and intestinal α-glucosidases is still unknown. It can be assumed that the compounds may form hydrogen bonds with the polar groups (amide, guanidine, peptide, amino, and carboxyl groups) of amino acid residues in the active site of intestinal α-glucosidase by covalent and/or non-covalent interaction. Further investigation is needed using X-ray crystallography and computer modeling to evaluate the binding activity of these compounds on the enzymes.

In general, α-glucosidases are classified into two families according to their substrate specificities. The first family consists of bacterial, yeast (*Saccharomyces cerevisiae*), and insect enzymes, named α-glucosidase I, which hydrolyze heterogeneous substrates or glucosyl structures, such as *p*-nitrophenyl α-glucoside[14,15]. Those from mold, plant, and mammalian tissues are called α-glucosidase II, and recognize maltooligosaccharides or maltosyl structures[16]. By analysis of the partial amino acid sequence containing the catalytic regions, the family I α-glucosidases are composed of four catalytic regions, whereas the family II α-glucosidases contain two catalytic regions responsible for the enzyme reaction[17]. The difference in structure of the catalytic regions on yeast and mammalian α-glucosidases may directly affect the binding affinity of cinnamic acid derivatives. Our recent published data showed that the presence of hydroxyl residue in cinnamic acid gave less potent yeast α-glucosidase inhibition than that of methoxyl residue at the same position[10]. In addition, it has been found that *p*-methoxycinnamic acid is the most potent inhibitor among cinnamic acid derivatives against yeast α-glucosidase. However, the present study revealed that *p*-methoxycinnamic acid was inactive in intestinal α-glucosidase inhibition. Our findings support that the inhibitor of yeast α-glucosidase may not necessarily inhibit small-intestinal α-glucosidases.

Ferulic acid, a potent intestinal sucrase inhibitor, is commonly found in fruits and vegetables such as potato[18], sweetcorn[19], and rice bran[20]. The daily intake for a person who has a regular consumption of cereal products can be up to 100 mg of ferulic acid[21]. Recent studies have reported that oral administration of ferulic acid (50 mg/kg) or the ethyl acetate fraction of rice bran (200 mg/kg) significantly decreases blood glucose levels and increases plasma insulin levels in type 2 diabetic mice by elevating hepatic glycogen synthesis and glucokinase activity[21]. From phytochemical analysis, the ethyl acetate fraction of rice bran contains a large quantity of phenolic acids (526 mg%), of which ferulic acid is the most abundant (303 mg%). Moreover, it has been reported that oral administration of rice bran (20 g/day for an 8-week period) decreases the levels of

glycosylated hemoglobin and fasting glucose and increases the serum insulin concentration in type 2 diabetic patients[22]. Isoferulic acid, an isomer of ferulic acid, is a main active ingredient of the rhizoma of *Cimicifuga beracleifolia*, which is used frequently in Japanese traditional medicine as an anti-inflammatory drug. It has been recently reported that isoferulic acid exhibits antihyperglycemic activity in streptozotocin (STZ)-induced diabetic rats[7]. It activates α_1-adrenoceptors to enhance the secretion of β-endorphin, which stimulates the opioid μ-receptors to increase glucose use and/or reduce hepatic gluconeogenesis, resulting in a reduction of the plasma glucose concentration[23]. Our previous studies demonstrated that ferulic acid, but not isoferulic acid, directly stimulates insulin secretion from the pancreas, leading to decreased plasma glucose concentrations in rats[24]. Caffeic acid is a natural phenolic compound widely distributed in plant-derived materials such as tea, wine, and olive oil. It is also found in many types of fruit, and coffee, in high concentrations, and has exhibited pharmacological properties such as antioxidant, anticancer, and antimutagenic activities[25]. On the basis of the caffeic acid content of brewed coffee measured after hydrolytic treatment, an intake of about 166 mg of caffeic acid can be calculated per cup of coffee[26]. Oral administration of caffeic acid decreases blood glucose and glycosylated hemoglobin levels in diabetic rats via its ability to enhance insulin secretion and to decrease hepatic glucose output along with the increased level of adipocyte glucose disposal in type 2 diabetic animals[27]. The abovementioned literature suggests that cinnamic acid derivatives act as insulin secretagogs and insulinomimetic agents. Considering the data obtained from this investigation, it is concluded that cinnamic acid derivatives contribute another mechanism in the control of hyperglycemia by inhibiting intestinal α-glucosidase, which contributes to the decrease of hemoglobin A_{1c} (HbA$_{1c}$). The decrease of HbA$_{1c}$ could reduce the incidence of chronic vascular complications in diabetic patients[3].

The combination of acarbose and ferulic acid, isoferulic acid, and caffeic acid could produce more effective intestinal sucrase inhibition. This may lead to a reduction of postprandial hyperglycemia and hyperinsulinemia in type 2 diabetes. Further *in vivo* experiments should be carried out in order to investigate the effect of the combination of these compounds and acarbose on lowering blood glucose in type 2 diabetes. In addition, the most common adverse effect of acarbose is a gastrointestinal disturbance induced by gas production upon fermentation of unabsorbed carbohydrates in the bowel[28]. Acarbose has rarely been associated with systemic adverse effects, but in some cases acute severe hepatotoxicity has been reported[29]. These adverse effects tend to increase with higher doses. Our findings suggest that the three compounds may enable a lower dose of acarbose to be used, resulting in diminishment of the adverse effect.

Although ferulic acid, isoferulic acid, and caffeic acid have been investigated regarding the possible mechanisms of antidiabetic activities, the present study shows that these compounds also inhibit intestinal α-glucosidase, which is one of the therapeutic approaches for treatment of diabetes mellitus. From this point of view, the intake of cinnamic acid derivative-enriched foods by diabetic patients may suppress postprandial hyperglycemia, helping in prevention of diabetic complications. Further comprehensive pharmacological investigations of these compounds are required to evaluate their toxicity and clinical efficacy for potential applications in the prevention and treatment of diabetes mellitus.

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