Original paper

Inhibitory Effect of Oligomeric Polyphenols from Peanut-skin on Sugar Digestion Enzymes and Glucose Transport

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Inhibitory effects of oligomeric polyphenols extracted from peanut skin on α -amylase, maltase, sucrase and glucose transport were investigated. Epicatechin-(4 β →6)-epicatechin-(2 β →O→7, 4 β →8)-catechin (EEC) as a procyanidin trimer inhibited α -amylase activity in a dose-dependent manner, while (+)-catechin and procyanidin A1 as a procyanidin dimer did not show any inhibitory activity up to 5 mg/mL. In the case of sucrase, EEC showed inhibitory activity stronger than (+)-catechin and procyanidin A1. Inhibitory effects of peanut skin on glucose transport in the small intestine were also investigated. The level of glucose transport in Caco-2 cells was significantly decreased by EEC compared to (+)-catechin. These results suggest that the inhibitory effects of procyanidins on α -amylase and α -glucosidase, and the suppression of intestinal glucose transport may change with the degree of polymerization.

Keywords: α -amylase, glucose transport, α -glucosidase, peanut skin, procyanidin

Introduction

The health contribution of non-nutritive components of crop origin is of global interest. Peanut skin, *Arachis hypogaea* L., is a rich source of polyphenols, containing approximately 1.4% by dry weight (Tomochika *et al.*, 2011). We have found that peanut skin polyphenols have anti-allergic and anti-hypercholesterolemia effects (Tamura *et al.*, 2013, Tamura *et al.*, 2012; Tomochika *et al.*, 2011) as well as antioxidative function (Verstraeten *et al.*, 2005). We identified procyanidin A1 as a procyanidin dimer and epicatechin-(4 β →6)-epicatechin-(2 β →O→7, 4 β →8)-catechin (EEC) as a procyanidin trimer from peanut skin (Fig. 1). The bioactivity of these compounds is closely related to their

stereochemistry. Our data showed that procyanidin A1 presented the inhibitory activity of β -hexosaminidase release, while (+)-catechin had no such activity. With respect to hypocholesterolemic effects, EEC had a more potent cholesterol micelle-degrading activity than procyanidin A1 *in vitro* (Tamura *et al.*, 2012). Other research has revealed that the anti-inflammatory and anti-melanogenic activities of procyanidin dimers and trimers from peanut skin were stronger than those of monomer and tetramers (Tatsuno *et al.*, 2012).

Polyphenols also exert hypoglycemic effects (Sowmya *et al.*, 2010). Serum glucose levels are affected by dietary carbohydrates, and thus maintenance of proper serum glucose concentrations is

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important for human health. In the small intestine, the digestion of carbohydrates is regulated by α -glucosidase, and the resulting monosaccharides are transported across intestinal cells via glucose transporters. It is well known that oral administration of tea-, fruit-, and medicinal plant-extracts with maltose and starch suppressed postprandial hyperglycemia in rats and humans (Sowmya *et al.*, 2010; Fadzelly *et al.*, 2006; Deguchi *et al.*, 2010; Ndong *et al.*, 2007). One of the reasons for this effect is that polyphenols inhibit α -amylase and α -glucosidase activities (Tsujita *et al.*, 2013; Adisakwattana *et al.*, 2011; Gu *et al.*, 2011; Ma *et al.*, 2010); another is that polyphenols inhibit glucose transport via its transporter (Ovádi *et al.*, 2005; Kobayashi *et al.*, 2000).

Meanwhile, almost all mechanistic studies on the hypoglycemic effect of polyphenols were conducted with polyphenol-containing extracts or monomeric polyphenols such as catechin, epicatechin, (-)-epigallocatechingallate, quercetin, and myricetin (Ovádi *et al.*, 2005). Little is known about the relationship between anti-hyperglycemic effects and degrees of polymerization (PD). In this study, we assessed the inhibitory activity of (+)-catechin, procyanidin A1 and EEC on α -amylase and α -glucosidase to elucidate the different potency of inhibitory activity in the intestine. Effects of these procyanidins on glucose absorption were also investigated.

Material and Methods

Preparation of peanut skin extracts and procyanidins Hot water soluble polyphenol fraction (peanut skin extract, PSE), procyanidin A1, and EEC were prepared from peanut skin as described previously (Tamura *et al.*, 2012, 2013; Tomochika *et al.*, 2011).

α -Amylase and α -Glucosidase Inhibition Assays Inhibition of α -amylase by peanut polyphenols was examined by the method of Kainuma *et al.* (1975) with modifications. α -Amylase was prepared from porcine pancreas (Sigma Aldrich Japan K.K., Tokyo, Japan), and suspended in 50 mM sodium acetate solution containing 5 mM calcium chloride. For the assay, 50 μ L of polyphenol sample (0–5 mg/mL water) was mixed with 150 μ L of α -amylase solution (2 U/mL), and the mixture was incubated at 37°C for 10 min. Then, 300 μ L of starch solution (0.4%) was added to the mixture and incubated for an additional 10 min. After heating at 100°C for 10 min to stop the degradation, the mixture was cooled on ice. Next, 100 μ L of 2 M sodium hydroxide solution and 200 μ L of 1% 3,5-dinitrosalicylic acid solution were added and heated in boiling water for 10 min. After cooling on ice, absorption was measured at 540 nm.

For the α -glucosidase inhibition assay, 1 g of rat intestinal acetone powder was homogenized with 9 mL of 56 mM maleate buffer (pH 6.0), followed by centrifugation at 1000 $\times$ g for 10 min at 4°C. The supernatant was used as the enzyme solution for the maltase and sucrase reactions. Fifty microliters of polyphenol sample (0–5 mg/mL water) was diluted with 50 μ L of maleate

buffer and added to 50 μ L of enzyme solution. After pre-incubation at room temperature for 10 min, 50 μ L of maltose or sucrose substrate solution (2% w/v in maleate buffer) was mixed in, and then incubated at 37°C for 30 and 60 min, respectively. The enzyme reaction was stopped by heating at 100°C for 10 min, and the glucose in the reaction mixture was measured using a commercial assay kit (Glucose CII-Test Wako, Wako Pure Chemical Industries).

The inhibitory effect of polyphenols was represented by the following equation:

$$\text{Inhibitory activity (\%)} = \frac{\{(A - B) - (C - D)\}}{A - B} \times 100 \dots \text{Eq. 1}$$

where A, B, C, and D are absorbance when distilled water was used in the assay instead of polyphenol, absorbance when distilled water and inactivated enzyme were used in the assay instead of polyphenol and intact enzyme, absorbance when polyphenol and intact enzyme were used in the assay, and absorbance when polyphenol and inactivated enzyme were used in the assay instead of intact enzyme, respectively.

Glucose transport measurements in Caco-2 cells Caco-2 cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum, 1% nonessential amino acids, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 50 μ g/mL gentamycin in a humidified atmosphere of 5% CO₂ at 37°C. Cells between passages 40 to 50 were used for the experiment. The transepithelial transport experiment was performed as described previously (Kobayashi *et al.*, 2007, 2013). Briefly, cells were cultured normally in 75-cm² tissue culture dishes to confluence and seeded onto 12-well transwell plates coated with type-I collagen. The cells were seeded at a density of 1 $\times$ 10⁵ cells /cm² and grown as a monolayer for 2 weeks. The monolayer cells were rinsed three times with Hanks' balanced salt solution (HBSS) and incubated in the same solution for 20 min at 37°C. The integrity of the cell layer was evaluated by measurement of transepithelial electrical resistance (TER) with Millicell-ERS equipment. A monolayer with a TER of > 200 Ω cm² was used. To measure the apical-to-basolateral permeability, 0.5 mL of HBSS (pH 7.4, 37°C) was added to the basolateral chamber of the transwell insert, and then test solution containing 1 mM glucose, 100 nM [1,2-³H(N)]-2-deoxy-D-glucose, (+)-catechin or procyanidins (60 μ g/mL) in HBSS buffer was added to the apical side. After a 15-min incubation at 37°C, 0.5 mL of the basolateral solution was collected and added to 5 mL of liquid scintillation cocktail Cleasol-II (Nacalai Tesque, Kyoto, Japan) and the emitted radiation of samples was counted using a Packard Liquid Scintillation Analyzer 1600TR. An untreated HEPES buffer containing 1 mM glucose was used as a control (blank value).

Statistical Analysis Values in a group were selected by Thompson's outlier test ($p < 0.05$). Between-group differences were detected by Tukey's multiple-range test following one-way analysis of variance (ANOVA). Statistical analyses were performed

Fig. 1. Structures of polyphenols purified from peanut skin. Structures of (+)-catechin (a), procyanidin A1 (b), and epicatechin-(4 β →6)-epicatechin-(2 β →O→7,4 β →8)-catechin (EEC) (c) are shown.

with SPSS software and each measurement was expressed as mean $\pm$ standard deviation. Different letters in the figures indicate significant differences ($p < 0.05$).

Results

Inhibitory activity of purified polyphenols from PSE on α -amylase and α -glucosidase PSE contained 4.73% polyphenol, and the PSE polyphenol contained 0.24% (+)-catechin, 5.62% procyanidin A1, and 2.20% EEC. We examined the inhibitory effect of polyphenols with different PD on α -amylase, maltase, and sucrase activities (Fig. 2a-c). EEC inhibited α -amylase activity dose-dependently, and showed 76.2% inhibition at 4 mg/mL. On the other hand, (+)-catechin and procyanidin A1 did not show any inhibitory activity up to 5 mg/mL (Fig. 2a). With respect to maltase, the three polyphenols of 3 to 5 mg/mL inhibited the enzymatic activity in the same ratio (Fig. 2b). Procyanidin A1 showed stronger inhibitory activity than EEC at lower concentrations (1 and 2 mg/mL). Sucrase activity was inhibited by all polyphenols in a dose-dependent manner up to 4 mg/mL (Fig. 2c). EEC showed significant strong inhibitory activity against sucrase. (+)-Catechin and procyanidin A1 showed 51.5 and 64.9% inhibition while EEC caused 89.1% inhibition at 5 mg/mL.

Inhibitory effect of peanut skin polyphenols on glucose transport We examined the level of glucose uptake in Caco-2 cells after treating the cells with (+)-catechin, procyanidin A1, and EEC. As shown in Fig. 3, the glucose uptake level was significantly decreased by the presence of polyphenols. It was found that the inhibitory effect of EEC was significantly higher than that of (+)-catechin. Glucose transport was suppressed with increasing PD, although no significant difference was observed between (+)-catechin and procyanidin A1, or procyanidin A1 and EEC.

Discussion

Peanut polyphenols with different structures showed distinct inhibitory activities (Fig. 4). Recently, Zhang *et al.* (2013) reported that epicatechin-(2 β →O→7, 4 β →8)-[catechin-(6→4 β)]-

Fig. 2. Inhibitory effects of polyphenols purified from peanut skin on α -amylase (a), maltase (b), and sucrase (c) activities. Inhibition of α -amylase (a), maltase (b), and sucrase (c) activities was measured. Open circle, (+)-catechin; filled circle, procyanidin A1; open triangle, EEC. Data represent means $\pm$ SD ($n = 3$). Different letters indicate significant differences ($p < 0.05$).

Fig. 3. Inhibitory effects of peanut skin polyphenols on glucose transport in Caco-2 cells

An HBSS test solution containing [1,2-³H(N)]-2-deoxy-D-glucose and polyphenol (60 µg/mL) was added to the apical side of the well. After 15-min incubation, scintillation counts of the basolateral solution were determined. Data represent means ± SD (n = 3). Different letters indicate significant differences (*p* < 0.05).

epicatechin has strong inhibitory effects on maltase, whereas epicatechin-(4β→8)-epicatechin-(2β→O→7, 4β→8)-catechin exhibited strong inhibition of sucrase. Our results showed that EEC inhibited α-amylase activity dose-dependently whereas (+)-catechin and procyanidin A1 had no such activity (Fig. 2a). Cocoa polyphenols are an example (Gu *et al.*, 2011) where (–)-epicatechin showed hardly any α-amylase inhibition activity whereas lower molecular weight compounds with a PD < 5 showed 15% inhibition at a concentration of 100 µM. Furthermore, high-molecular-weight procyanidins with a PD ranging from 5 to 10 inhibited α-amylase by 17–45.5% at 100 µM; thus, Gu *et al.* (2011) proposed that the inhibition of α-amylase activity was dependent on the PD. In our study, procyanidin A1 with a PD of 2 did not inhibit α-amylase activity at a concentration of 4 mg/mL, as observed for (+)-catechin. On the other hand, maltase and sucrase were inhibited by (+)-catechin, procyanidin A1, and EEC. Especially, sucrase was significantly inhibited by EEC (Fig. 2c). A similar result was reported for procyanidins from *Aronia melanocarpa* (Bräunlich *et al.*, 2013) and *Cynomoriaceae* (Ma *et al.*, 2010), where α-glucosidase inhibition by flavan-3-ol monomer and oligomers increased as the molecular weight increased; further, a significant difference in potency between the strongest ones (pentamers) and the weakest one (monomer) was observed. Jeon *et al.* (2013) also showed that catechin polymers produced by laccase and present as a mixture of dimers, trimers, and tetramers, suppressed the rise in blood glucose more than the catechin monomer. These results suggest that sucrase activity is increasingly inhibited as PD increases. As PD increases, the number of hydroxyl groups, to which proteins containing digestion enzymes may bind, increases. Future studies are needed to elucidate the enzyme inhibition mechanism from the interaction between the enzyme and the polyphenol oligomer.

It has also been investigated whether various polyphenols show

Fig. 4. Proposed mechanism for the suppression of intestinal glucose absorption by peanut polyphenols

The suppression of the postprandial blood glucose level is considered to involve both the inhibition of α-amylase and α-glucosidase as well as the suppression of intestinal glucose transport. C: (+)-catechin, PA1: procyanidin A1. Magnitude of the suppression is indicated by equal and inequality signs.

different degrees of inhibition of glucose transport in the small intestine. Transepithelial transport of polyphenols in Caco-2 cells has been extensively investigated. (+)-Catechin and procyanidin dimer and trimer showed similar permeability coefficients, in contrast, the permeability of a procyanidin polymer with an average PD of 6 (molecular weight 1,740) was ~10 times lower (Deprez *et al.*, 2001). On the other hand, Kobayashi *et al.* (2000) reported that (–)-epicatechin gallate binds to the glucose transporter as an antagonist-like molecule and inhibits the glucose transport. They proposed that the galloyl ester group is essential for this inhibitory activity. Little is known of the relationship between the PD of polyphenols and the inhibition of transport. Glucose uptake by insulin-sensitive glucose transporter (GLUT4) in myotubes was significantly enhanced by treatment with procyanidin tetramers, whereas cyaniding 3-glucoside, procyanidin dimer, and trimer had no effect on the level of glucose transport (Kobayashi *et al.*, 2000). In the present study, EEC as well as procyanidin A1 and (+)-catechin possess inhibition activity toward glucose transport, and the inhibition was increased as the PD increased (Fig. 3).

This is the first report of the relationship between the degree of potency of glucose transport inhibition and the PD. This paper also reported the different inhibitory activities of peanut skin procyanidins on carbohydrate digestive enzymes. Further analysis of the structural interaction between polyphenols and glycolytic enzymes, or polyphenols and glucose transporters would aid in the elucidation of the mechanism of polyphenols in hyperglycemia and in the development of new functional food components.

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