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Preparation and characterisation of peanut seed skin polyphenols

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ABSTRACT

Using α-amylase inhibition as a separation guide, polyphenolic compounds from peanut seed skin were prepared. During preparation, specific α-amylase inhibitory activities were increased about 4-fold. High-resolution MALDI-TOF mass spectra showed that the structure of this sample was a series of polyflavan-3-ols, up to 15-mer, composed of catechin/epicatechin units together with several afzelechin/epiafzelechin units and gallocatechin/epigallocatechin units. The observed precious mass values suggest that the polymers consist of both interflavanoid C–C linkages (A-type) and interflavanoid ether linkages (B-type). Oral administration of the polyphenol fraction to rats fed corn starch significantly suppressed an increase in blood glucose levels in a dose dependent manner. Administration of the polyphenol fraction to rats fed maltose or sucrose delayed the increase in blood glucose levels. These results suggest peanut seed skin contains polyphenols with strong α-amylase inhibitory activity, which retard absorption of carbohydrates and mainly function through inhibition of α-amylase.

1. Introduction

The peanut (*Arachis hypogaea* L.) is not in fact a nut, but a species of the legume family which originated in South America. It is an annual herbaceous plant which grows 30–50 cm in height and is cultivated around the world in tropical, subtropical and warm temperate regions. Peanuts are comprised of kernels, skins and hulls. Peanut skins and hulls are generated in large quantities as waste products during industrial seed skin-free kernel processing. The phenolic compounds in peanuts are mainly located in the skins and hulls (Yen, Chang, & Duh, 2005) and although the weight of the skin is small in relation to the total nut, it contains a large ratio of the total polyphenols present in the nut. Nepote, Grosso, and Guzman (2005) reported that the content of phenolic compounds in peanut skin was 115–149 mg/g dry skin, depending on the solvent used. Yu, Ahmedna, and Goktepe (2005) reported that the total phenolics measured about 90–125 mg/g dry skin. The phenolic compounds are not essential for the survival of the plant and it is suggested that these compounds may protect the plant against environmental stresses. They are known to be useful in formulating nutritional or medicinal supplements for the treatment of several diseases. Some investigators reported that peanut seed skin polyphenols have an antioxidant capacity and hypolipidemic effects (Bansode, Randolph, Hurley, & Ahmedna, 2012; Lou et al., 2004; Tamura et al., 2012). One of the important activities of polyphenols is the inhibition of digestive enzymes, especially carbohydrate-hydrolysing enzymes such as α-amylase and α-glucosidase. Xiao, Ni, Kai, and Chen (2013) reviewed reports on the structure–activity relationship of polyphenols inhibiting α-amylase. We have investigated the inhibitory effects of nut polyphenols against α-amylase. *In vitro* studies have shown that many nut polyphenols inhibited carbohydrate-hydrolysing enzymes, including polyphenols from chestnut (Tsujita et al., 2011), Japanese house chestnut (Ogawa et al., 2008), almond (Tsujita, Shintani, & Sato, 2013), evergreen mangrove seeds (Gowri, Tiwari, Ali, & Rao, 2007) and berry (McDougall and Stewart, 2005). In this paper, we focused on the separation and characterisation of polyphenols from peanut seed skin. We also focussed on the effects of the separated materials on α-amylase activity and carbohydrate absorption.

2. Materials and methods

2.1. Materials

Roasted peanut (*Arachis hypogaea*) seed skin was obtained from Chuon Co., Ltd. (Matsuyama, Japan). α-Amylases from porcine pancreas, pancreatic lipase (Type VI-S) from porcine pancreas, intestinal acetone powder from rat, Folin–Ciocalteu reagent, (+)-catechin and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma–Aldrich Japan (Tokyo, Japan). Cyanidin chloride was obtained from ChromaDex (Irvine, CA). Trolox was obtained from Calbiochem (Darmstadt, Germany). Sephadex LH-20 was obtained

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from GE Healthcare Japan (Tokyo, Japan). Ultrafilters were obtained from ADVANTEC (Tokyo, Japan). Octa-decyl silyl silica gel (ODS) was obtained from Yamazen Co. (Osaka, Japan).

2.2. Separation of polyphenol from peanut seed skin extract

Ten grammes of roasted and milled peanut seed skins were added to 70% aqueous acetone (100 ml), followed by shaking at 40 °C for 24 h. The mixture was filtered through gauze layers and the filtrate was concentrated under reduced pressure and lyophilised to generate the acetone extract. The acetone extract (2 g) was added to 1 l of 70% aqueous ethanol, followed by stirring at 40 °C for 1 h. The mixture was centrifuged at 3000*g* for 10 min and the supernatant was fractionated stepwise using Ultrafilters (Dia: 90 mm; molecular weight cut off 200, 50, and 10 kDa). Each fraction was concentrated under reduced pressure and lyophilised. The fraction over 200 kDa (300 mg) was dissolved in 200 ml of water before being applied to a Sephadex LH-20 column (300 mm × 35 mm i.d.) equilibrated with water. The adsorbed compounds were eluted stepwise with 500 ml of 30%, 40%, 50%, 60% and 80% aqueous acetone after washing with water (1 l). The eluates were concentrated under reduced pressure and lyophilised. The 50–60% aqueous acetone fraction (100 mg) was dissolved in 100 ml of water before being applied to an ODS column (175 mm × 18 mm i.d.) equilibrated with water. The active compounds were eluted as a single peak with a linear gradient of 20–50% aqueous methanol after washing with water (100 ml) and 20% aqueous methanol (100 ml). The eluate, containing high α-amylase inhibitory activity, was concentrated and lyophilised. During separation, α-amylase inhibition and the amount of polyphenol were monitored.

2.3. Assay methods

α-Amylase activity was determined by measuring the reducing power of released oligosaccharide from soluble starch using the method of Miller (1959), with the following minor modifications (Tsujita, Takaku, & Suzuki, 2008): the assay system consisted of 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 in a total volume of 1 ml. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 0.1 ml of 2 N NaOH and 0.1 ml of colour reagent (4.4 μmol of 3,5-dinitrosalicylic acid, 106 μmol of potassium sodium (+)-tartrate tetrahydrate and 40 μmol of NaOH), followed by 3 min incubation at 100 °C and subsequent absorbance measurement at 540 nm.

Maltase and sucrase activities were determined using maltose and sucrose as the substrates, respectively, and glucose produced in the reaction was measured with a commercial assay kit (Glucose C II-test, Wako Pure Chemical Industries, Ltd., Osaka, Japan) (Dahlqvist, 1964; Kessler et al., 1978). Pancreatic lipase activity was determined by measuring the rate of release of oleic acid from trioleoylglycerol (Tsujita, Matsuura, & Okuda, 1996). The rate of enzyme inhibition was calculated as a percentage of the control (without inhibitor) using the following formula:

$$\text{Enzyme inhibition } (\%) = [(A_O - A_i)/A_O] \times 100$$

where: A_i = activity with inhibitor; A_O = control activity (activity without inhibitor).

One unit of enzyme inhibition (U) was expressed by the weight of the IC_{50} (concentration of inhibitor to inhibit 50% of its activity) value per ml.

Polyphenols (total phenolics) were determined using the method of Folin–Ciocalteu and (+)-catechin as the standard (Julkunen-Tiitto, 1985). In addition, flavan-3-ol concentration was determined using the vanillin assay (Sun, Ricardo-de-Silva, & Spranger, 1988) and procyanidins were determined using the butanolic-HCl assay (Porter, Hrstich, & Chen, 1986) using (+)-catechin and cyanidin chloride as standards, respectively.

Antioxidant activities were determined using the DPPH radical scavenging capacity assay. The DPPH radical scavenging capacity of each fraction was determined according to the method of Miliauskas, Venskutonis, and Van Beek (2004). Radical scavenging activity of the samples or trolox calibration solutions against stable DPPH radicals was determined spectrophotometrically and the activities were expressed as μmol trolox equivalents (TE)/mg sample.

2.4. Analysis of polyphenol by MALDI-TOF MS

The sample (0.5 mg of the purified fraction) was dissolved in 0.5 ml of acetone. About 10 mg of 2,4,6-trihydroxyacetophenone (THAP, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a matrix and dissolved in 1 ml of acetone/Milli-Q water at a ratio of 4/1 (v/v). The sample and matrix solutions were then mixed at a ratio of 10/1 (v/v), and 1 μl was then placed on a target plate and dried in air. MALDI-TOF mass spectra were collected on a JMS-S-3000 MALDI Spiral TOF mass spectrometer (JEOL Ltd., Tokyo, Japan). Ions generated by irradiation with a 349 nm Nd:YLF laser were accelerated at 20 kV. The ions then passed along a spiral ion trajectory with a flight length of approximately 17 m. The settings of delay time and grid voltage were optimised to maintain a constant, at ΔM = 0.02–0.03 Da over the range of m/z 800–3000. Mass calibration was performed using a poly(methyl methacrylate) (PMMA) standard (peak-top molecular weight, M_p = 1310) purchased from Polymer Laboratories (Church Stretton, UK). Three mass spectra for each sample were collected.

2.5. 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 250–300 g, were starved overnight (15 h) and divided into two groups. The test group received 2.5 ml of carbohydrate solution (2 g/kg body weight) containing the peanut seed skin polyphenols while the control group received carbohydrate solution only. The solutions were fed via a stomach tube. 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) (Tsujita et al., 2008). After a 1 week interval, the test and control groups were switched and the experiment was repeated.

2.6. Statistical analysis

Results are expressed as the mean + S.E. Statistical significance of differences, with and without (control) the samples, were assessed using the paired Student's *t*-test.

3. Results and discussion

Roasted peanut seed skin was extracted with water and aqueous organic solvent (70% aqueous methanol, ethanol, acetone or acetonitrile). We found that aqueous acetone was the most effective solvent, producing the highest freeze-dried weight, total polyphenol content and α-amylase inhibitors activity (the specific α-amylase inhibitory activities of aqueous methanol, ethanol, acetone and acetonitrile were 28.5, 22.4, 32.4 and 24.9 U/mg dry weight, respectively). α-Amylase inhibitors were not effectively extracted with non-polar solvents such as hexane, ethyl acetate and chloroform: the specific α-amylase inhibitory activities were