JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 12431–12439 12431
DOI:10.1021/jf102313b

Fatty Acids, Epicatechin-Dimethylgallate, and Rutin Interact with Buckwheat Starch Inhibiting Its Digestion by Amylase: Implications for the Decrease in Glycemic Index by Buckwheat Flour

Umeo Takahama*,† and Sachiko Hirota‡

†Department of Bioscience, Kyushu Dental College, Kitakyushu 803-8580, Japan, and ‡Department of Nutritional Science, Kyushu Women's University, Kitakyushu 807-8586, Japan

Glycemic indexes of bread made from mixtures of wheat flour and buckwheat flour are lower than those made from wheat flour. To discuss the mechanism of the buckwheat flour-dependent decrease in glycemic indexes, the formation of a starch−iodine complex and amylase-catalyzed digestion of starch were studied using buckwheat flour itself and buckwheat flour from which fatty acids, rutin, and proanthocyanidins including flavan-3-ols had been extracted. Absorbance due to the formation of a starch−iodine complex was larger in extracted than control flour, and starch in extracted flour was more susceptible to pancreatin-induced digestion than starch in control flour. Fatty acids, which were found in the buckwheat flour extract, bound to amylose in the extracted flour, inhibiting its digestion by pancreatin. Rutin and epicatechin-dimethylgallate, which were also found in the extract, bound to both amylose and amylopectin in the extracted flour, inhibiting their digestion induced by pancreatin. We discussed from these results that the lower glycemic indexes of bread made from mixtures of wheat flour and buckwheat flour were due to binding of fatty acids, rutin, and epicatechin-dimethylgallate, which were contained in buckwheat flour, to wheat flour starch.

KEYWORDS: Amylase resistance; formation of starch−polyphenol complexes; inhibition of starch−iodine complex formation; spectrophotometric study

INTRODUCTION

Buckwheat (*Fagopyrum esculentum* Möench) is widely cultivated in North America originating from East Asia and is mainly consumed in Central European countries, the United States, Canada, and Asian countries as a constituent of foods such as pancakes, noodles, and buckwheat bread blended with flour from other cereals. Recently, buckwheat flour has been introduced in many countries because the flour is nutritionally rich and foods prepared using the flour have beneficial effects on human health. One of the beneficial effects is the decrease in glycemic and insulin indexes (*1*).

Buckwheat flour contains 70−91% (w/w) of starch depending on the flour types, and the starch consists of about 25% amylose and 75% amylopectin (*2*). It is known that the glycemic indexes of foods prepared from buckwheat flour are lower than those prepared from wheat flour. For example, the glycemic indexes of boiled buckwheat groats and bread prepared from the same amount of wheat flour and buckwheat flour are 61 and 66, respectively (*1*). The glycemic index of bread prepared from whole wheat flour is approximately 70. The decrease in glycemic indexes by buckwheat flour is supported by the data of insulin indexes; the insulin indexes of boiled buckwheat groats and bread prepared from the same amount of wheat flour and buckwheat flour are 53 and 74, respectively (*1*). Therefore, foods prepared from buckwheat flour and groats have a potential use for diabetics. The decrease in glycemic and insulin indexes can be attributed to the formation of amylase-resistant starch by heating (*1*−*4*).

Although buckwheat flour starch becomes amylase-resistant after heating, it has not yet been fully understood how the amylase resistance is acquired. The acquisition of amylase resistance can be attributed to the formation of starch−lipid complexes because buckwheat starch contains lipids (*5*, *6*). It has been reported that lipids and free fatty acids can inhibit amylase-catalyzed digestion of starch by forming starch−lipid or −fatty acid complexes (*7*−*11*). On the other hand, buckwheat flour contains rutin and proanthocyanidins including flavan-3-ols (*12*, *13*). These flavonoids are also candidates to form the amylase-resistant starch by heating because polyphenols can make complexes with not only starch (*14*, *15*) but also various polysaccharides noncovalently (*16*−*21*). Furthermore, polyphenols including flavonoids have been reported to be inhibitors of α-amylase activity (*22*−*27*). This paper mainly deals with the interactions between buckwheat starch and fatty acids, rutin, or epicatechin-dimethylgallate, which was isolated from in buckwheat flour, and the inhibition of amylase-catalyzed digestion of starch by the above components.

MATERIALS AND METHODS

Reagents. Pancreatin from hog pancreas, rutin, quercetin, and fatty acids (myristic, palmitic, stearic, oleic, and linoleic acids) was obtained

*To whom correspondence should be addressed. E-mail: takahama@kyu-dent.ac.jp.

Published on Web 11/08/2010

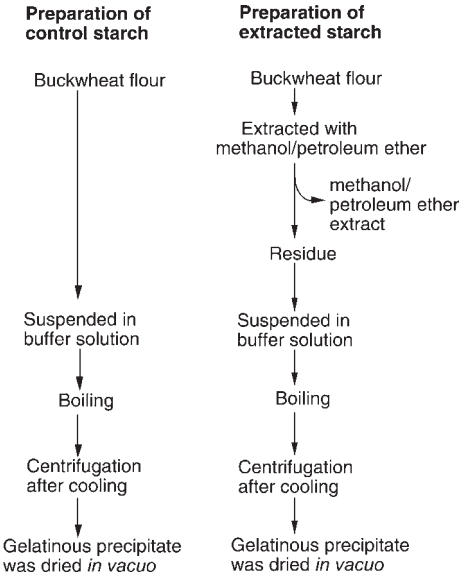

Figure 1. Scheme describing the preparation of control starch and extracted starch.

from Wako Pure Chem. Ind. (Osaka, Japan). Linolenic acid, amylose, and amylopectin were obtained from Tokyo Kasei Ind. (Tokyo, Japan). 4-Hydroxybenzhydrazide or *p*-hydroxybenzoic acid hydrazine was from Sigma-Aldrich Japan (Tokyo, Japan). Buckwheat flour (cultivar Mancan) was from Itsuki Shokuhin (Kumamoto, Japan).

Preparation of Iodine and Pancreatin Solutions. Potassium iodide (1.5 g) was dissolved in 12.5 mL of water. Iodine (0.635 g) was added to the solution, and the volume of the mixture of potassium iodide and iodine was brought to 50 mL by adding water. The solution (equivalent to 100 mM iodine) was kept in the dark to use as iodine solution. Pancreatin (10 mg/mL) was dissolved in water.

Preparation of Extracted Buckwheat Flour Starch. Buckwheat flour (0.3 g) was extracted with 5 mL of methanol three times and then extracted with 5 mL of petroleum ether twice (**Figure 1**). The extracted buckwheat flour and nonextracted flour were suspended each in the mixture of 3 mL of 50 mM KCl-HCl (pH 1.76) and 7 mL of water. The suspensions, the pH of which was 4.6, were heated with a microwave oven (800 W) until it boiled, and the microwave oven was turned off immediately after the start of boiling. The reason that buckwheat flour was suspended in the acidic buffer solution was to suppress autoxidation of phenolic compounds during heating. The boiled buckwheat suspensions were cooled to room temperature and centrifuged at 3500*g* for 1 min. Gelatinous starch sediments were obtained. The sediments were dried in vacuo over NaOH. In the following, dried starch preparations prepared from nonextracted and extracted buckwheat flour were referred to as control and extracted starch, respectively.

The methanol/petroleum ether (MP) extracts of buckwheat flour were combined, and the organic solvents were evaporated with a rotary evaporator. The residue was dissolved in 1 mL of methanol, referred to as MP extract, and used to study its effects on the formation of the starch−iodine complex and amylase-catalyzed digestion of starch. Fatty acids and phenolic components in MP extract were separated by high-performance liquid chromatography (HPLC) using a Shim-pack CLC-C_8 column (6 mm i.d. × 15 cm) (Shimadzu). The mobile phase used to separate fatty acids, which were detected at 210 nm, was a mixture of acetonitrile and 0.1% (v/v) phosphoric acid (2:1, v/v). Proanthocyanidins including flavan-3-ols and rutin, which were detected at 275 and 350 nm, respectively, were separated using a mobile phase of a mixture of methanol, water, and acetic acid (36:54:1, v/v). The flow rate of the mobile phases was 1 mL/min.

Preparation of Starch Suspensions. Each control starch and extracted starch (5 mg) were suspended in 1 mL of water. The suspensions were kept for 30 min at room temperature to make starch swell by absorbing water, and then, 7.5 mL of 100 mM sodium phosphate (pH 7.1) and 7.5 mL of 50 mM KCl−HCl (pH 2.0) were successively added to the suspensions. In other experiments, 7.5 mL of 50 mM KCl−HCl (pH 2.0) was added to the starch suspensions to simulate the conditions in the stomach after the ingestion of buckwheat starch. The acidic suspension was incubated for 30 min, and then, 7.5 mL of 100 mM sodium phosphate (pH 7.1) was added. The pH of the starch suspensions after the addition of acidic and neutral buffer solutions was 6.8. The suspensions were homogenized using a homogenizer and then sonicated for 10 s to use as control-starch and extracted-starch suspensions. The concentration of starch in the suspensions was 0.31 mg/mL. Amylose and amylopectin (10 mg/mL) were suspended in gently boiling water.

Formation of Starch−Iodine Complex. It is known that the starch−iodine complex can be used to estimate the concentration of starch and that the color of amylose−iodine complex (blue: λ_{max} = 560−660 nm) is different from that of the amylopectin−iodine complex (purple: λ_{max} = 500−520 nm) (*28*−*32*). Then, the formation of the starch−iodine complex was studied by recording the absorption (*A*) spectra from 900 to 500 nm using a 557 double-beam spectrophotometer (Hitachi, Tokyo, Japan). The light path of the measuring beam was 4 mm. When control starch and extracted starch were used, starch−iodine complexes were formed by adding 0.1 mL of iodine solution, which had been prepared as described above, to 0.9 mL of a starch suspension. When amylose and amylopectin were used, 30 μL of amylose suspension (equivalent to 0.3 mg of amylose) and 20 μL of amylopectin suspension (equivalent to 0.2 mg of amylopectin) were added each to 0.9 mL of the mixture of 50 mM KCl−HCl (pH 2.0) and 100 mM sodium phosphate (pH 7.1) (1:1, v/v), and then, 0.1 mL of iodine solution was added.

Effects of MP extract and reagents on the formation of the starch−iodine complex were studied as follows. A suspension of starch was added by MP extract or a reagent and incubated for 0.5 min to add 0.1 mL of iodine solution described above. Immediately after the addition of iodine solution, measurements of *A* spectra were started.

Measurement of Amylase Activity. Pancreatin-induced digestion of starch was studied using the above spectrophotometer. Pancreatin (10 μg) was added to 0.9 mL of a starch suspension described above. After incubation of the suspension for defined periods at 25 °C, 0.1 mL of iodine solution, which had been prepared as described above, was added. By the addition of iodine solution, pancreatin-induced starch digestion was terminated. Immediately after the addition of iodine solution, measurements of *A* spectra were started. The degree of the digestion was estimated from difference (ΔA) spectra after and before the digestion.

In addition, pancreatin-induced digestion of starch was also studied using 4-hydroxybenzhydrazide (*33*). Solution I was 0.33 M 4-hydroxybenzhydrazide dissolved in 0.6 M HCl, and solution II was a mixture of 0.042 M sodium citrate, 0.007 M $CaCl_2$, and 0.5 M NaOH in water. The mixture of solutions I and II (1:9, v/v) was prepared daily and stored on ice between uses. A control or extracted starch suspension (0.5 mL) was digested by pancreatin (10 μg/mL) for defined periods as described above. The reaction was terminated by adding 2.5 mL of the mixture of solutions I and II and incubated in boiling water for 6 min. After precipitates were cooled and removed by centrifugation, *A* at 410 nm was determined.

Isolation of Component X. Buckwheat flour (50 g) was suspended in 200 mL of 50 mM KCl−HCl (pH 1.76). The pH of the suspension was approximately 4. Ethyl acetate (300 mL) was added to the suspension and incubated for 1 h. After centrifugation for 3 min at 3500*g*, the ethyl acetate layer was collected and washed with water four times. The washed ethyl acetate layer was dehydrated with anhydrous sodium sulfate, and ethyl acetate was evaporated with a rotary evaporator. The residue was dissolved in 10 mL of methanol. The methanol solution was extracted with petroleum ether, and methanol was evaporated in vacuo. The residue was dissolved in 1 mL of methanol. The above extraction procedures were repeated four times, and the methanol solutions were combined. Methanol in the combined methanol solutions was evaporated with a rotary evaporator, and the residue was dissolved in 1 mL of methanol. Component X was prepared by preparative HPLC (see below).

Prior to its preparation, components contained in the methanol solution were analyzed using a Shim-pack CLC-ODS column (6 mm i.d. × 15 cm). The mobile phase was a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v), the pH of which was adjusted to 3.0 by adding 1 M HCl. The flow rate of the mobile phase was 1 mL/min.

Preparative HPLC was performed using a Shim-pack PREP-ODS column (20 mm i.d. × 25 cm). After a sample (0.2 mL) was applied, HPLC was performed using a mixture of methanol and 25 mM KH_2PO_4 (pH 3.0) (1:2, v/v) for about 45 min at a flow rate of 9 mL/min and then performed using methanol at a flow rate of 9 mL/min. Component X was found in a

methanol fraction. The preparative HPLC to collect the fraction of component X was repeated five times. The collected component X fractions were combined, methanol was evaporated with a rotary evaporator, and the residue was dissolved in 1 mL of methanol. An aliquot (0.2 mL) of the methanol solution was applied to the above preparative column to purify component X further. The mobile phase was a mixture of methanol and water (1:1, v/v), and the flow rate was 8 mL/min. The preparative HPLC was repeated five times. Fractions of component X were combined, the eluent was evaporated using a rotary evaporator, and the residue was dissolved in 1 mL of methanol to be used as component X.

LC/MS. An electrospray ionization mass spectrum of component X was obtained with a 3200 Q TRAP mass spectrometer (AB Sciex Instruments). Ionization was performed with an APCI probe voltage of −4.5 kV. HPLC for LC/MS was performed using a Shim-pack CLC-ODS column (15 cm × 6 mm i.d.) and a mixture of acetonitrile and 5 mM ammonium acetate, the pH of which was adjusted to 4.5 by acetic acid (2:3, v/v), as a mobile phase. The flow rate of the mobile phase was 1 mL/min.

Presentation of Data. Each experiment was repeated at least three times, and essentially, the same results were obtained. Typical data were presented as figures.

RESULTS AND DISCUSSION

Formation of Starch−Iodine Complex and Starch Digestion in Extracted Starch. Figure 2 shows *A* spectra of starch−iodine complexes. *A* of the control starch−iodine complex was smaller than that of extracted starch−iodine complex in the wavelength range from 500 to 900 nm. This result indicates that the concentration of starch, which could bind to iodine, was increased by the extraction of buckwheat flour with methanol and petroleum ether because the concentration of extracted starch was the same as that of control starch. The increase might be due to the extraction of fatty acids, proanthocyanidins including flavan-3-ols, and flavonols. Δ*A* spectrum of extracted minus control starch−iodine complexes had a broad peak at 567 nm. This result suggests that the concentration of amylose was mainly increased by the extraction because amylose−iodine complexes had *A* peaks at 540−660 nm (*28*−*32*).

Control (left panel) and extracted (right panel) starch suspensions were incubated for 0, 2, 4, 6, 8, and 30 min in the presence of pancreatin, and then, iodine solution was added (**Figure 3**). The formation of the starch−iodine complex in control starch and extracted starch decreased with the incubation time (downward arrows, progress of incubation time), indicating that both control starch and extracted starch were digested by amylase contained in pancreatin and that extracted starch was digested faster than control starch. Typical Δ*A* spectra between pancreatin-treated and -untreated starch (left panel, inset) indicate (i) that amylopectin was preferentially digested in control starch (A) and (ii) that not only amylopectin but also amylose was digested in extracted starch (B). Time courses of *A* decrease at 620 nm (right panel, inset) and indicate (i) that the rate of starch digestion was faster and (ii) that the amount of starch digested during 30 min of incubation was larger in extracted (●) than in control (○) starch.

Pancreatin-induced digestion of starch was also studied by measuring the production of reducing sugars (**Figure 4**). Their production was faster and larger in extracted starch (●) than in control starch (○), and time courses of the sugar production were similar to the decreases in the starch−iodine complex formation measured at 620 nm (**Figure 3**, right panel, inset). The results indicate that starch−iodine complex formation could be used to estimate the starch digestion under the conditions of this study.

Acid Treatment and Methanol Extraction of Control Starch. Ingested starch is mixed with gastric juice in the stomach. Then, the formation of a starch−iodine complex and pancreatin-induced digestion were studied using acid-treated control and extracted starch (see the Materials and Methods). The *A* value of the starch−iodine complex was smaller in control starch than

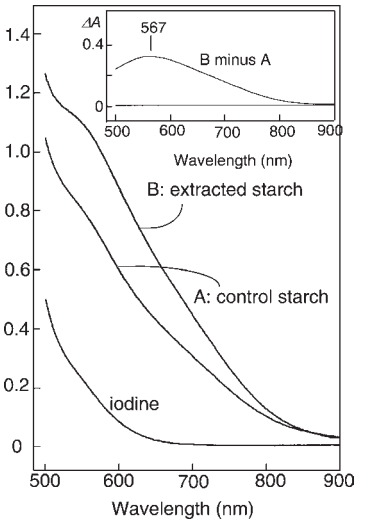

Figure 2. Absorption spectra of starch−iodine complexes. A, control starch; B, extracted starch. Inset: Δ*A* spectrum of B minus A.

extracted starch in the wavelength range from 500 to 900 nm (cf. **Figure 2**). Δ*A* spectra after and before pancreatin-induced digestion had a peak at approximately 530 nm in control starch and approximately 560 nm in extracted starch, and the *A* decrease at 620 nm by the digestion was approximately 1.8-fold faster in extracted starch than control starch. These results indicate that normal buckwheat starch would be digested slowly in the intestine after passing through the stomach.

Control starch (5 mg), which had been prepared as shown in **Figure 1**, was extracted with 5 mL of methanol and then suspended in the mixture of 50 mM KCl−HCl (pH 2.0) and 100 mM sodium phosphate (pH 7.1) (1:1, v/v) to study the starch−iodine complex formation and pancreatin-induced digestion. *A* due to the the formation of the starch−iodine complex was increased by methanol extraction in the wavelength range from 500 to 900 nm, and pancreatin-induced digestion was enhanced by methanol extraction (data not shown). These results and results in **Figures 2**−**4** indicate that components, which were extractable from buckwheat flour with methanol or MP, suppressed the formation of the starch−iodine complex and inhibited pancreatin-induced starch digestion. Hence, we studied the effects of MP extract on the formation of the starch−iodine complex and the pancreatin-induced digestion of starch because components of MP extract would be similar to those of methanol extract.

Effects of MP Extract on Starch−Iodine Complex Formation and Starch Digestion. MP extract suppressed the formation of the starch−iodine complex in extracted starch (**Figure 5**). Δ*A* spectra between the presence and the absence of MP extract showed that the suppression was increased with the increase in the amount of MP extract added (upper panel). The Δ*A* spectra also show that MP extract preferentially suppressed the formation of the amylose−iodine complex when the amount of MP extract added was small but suppressed the formation of the amylopectin−iodine complex as well as the amylose−iodine complex when the amount added was large. MP extract did not significantly affect the formation of the starch−iodine complex in control starch (data not shown), indicating that control starch was saturated with components contained in MP extract.

Figure 5 (lower panel) shows MP extract-dependent inhibition of pancreatin-induced digestion of extracted starch. The peak of Δ*A* spectra after and before the digestion shifted from 552 to 532 nm by addition of MP extract, and the Δ*A* at 532 nm decreased with the increase in the amount of MP extract added,

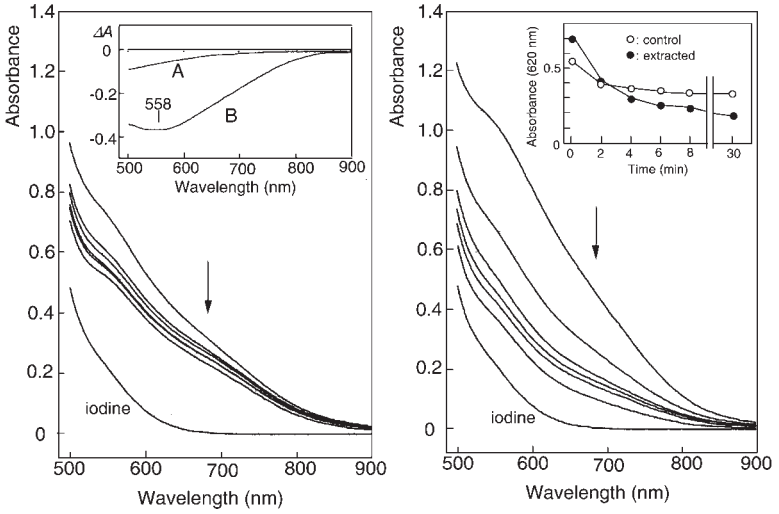

Figure 3. Pancreatin-induced digestion of starch. Downward arrows: Progress of incubation time (0, 2, 4, 6, 8, and 30 min) from top bottom trace. Left panel, control starch; right panel, extracted starch. Inset in the left panel: ΔA spectra after and before 2 min of pancreatin treatment. A, control starch; B, extracted starch. Inset in the right panel: A decrease at 620 nm. ○, control starch; and ●, extracted starch.

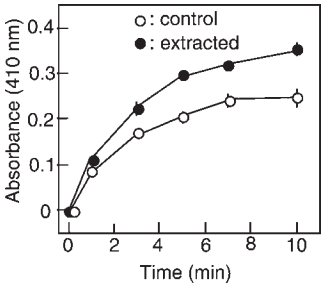

Figure 4. Pancreatin-induced production of reducing sugars. ○, control starch; ●, extracted starch. Each data point represents the average with SD (n = 3). In several data points, deviations were within circles.

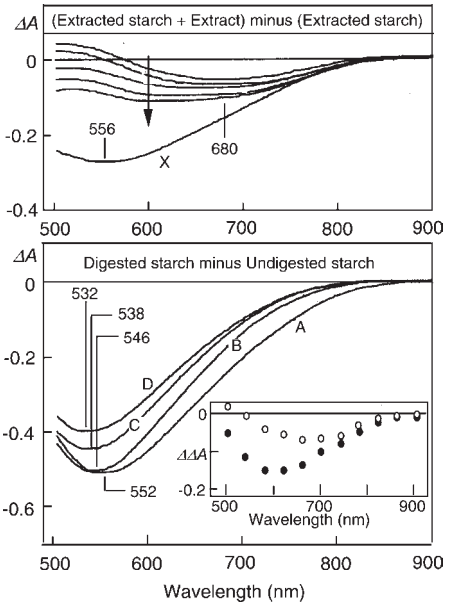

Figure 5. Effects of MP extract on the formation of the starch−iodine complex and starch digestion. Top panel, ΔA spectra before and after addition of various amounts of PM extract to extracted starch. A downward arrow, increase in the amount of added buckwheat flour extract (from top to bottom: 0.5, 1.0, 10, 20, and 30 μL/mL of extracted starch). X, ΔA spectrum of control minus extracted starch. Bottom panel, ΔA spectra after and before 2 min of pancreatin treatment. A, 0; B, 5; C, 10; and D, 30 μL of MP extract/mL of extracted starch. Inset: $\Delta\Delta A$ spectra. ○, A minus B; ●, A minus D.

indicating that addition of large amounts of MP resulted in the inhibition of not only amylose but also amylopectin digestion. This idea was supported by the shift of peaks of $\Delta\Delta A$ spectra, which were obtained by subtracting trace B from trace A (○) and trace C from trace A (●) in ΔA spectra, from approximately 680 to 600 nm with the increase in amount of MP extract added. Digestion of control starch by pancreatin was not significantly affected by MP extract (data not shown). This result supports the idea that control starch was saturated with components contained in MP extract and implies that MP extract did not affect amylase activity.

Because MP extract contained free fatty acids such as palmitic, stearic, oleic, linoleic, and linolenic acids at about 8.9, 0.23, 16, 23, and 1.4 μmol/g of buckwheat flour, effects of these components on the formation of the starch−iodine complex and pancreatin-induced digestion of starch were studied. Furthermore, MP extract contained rutin and proanthocyanidins including flavan-3-ols judging from their A spectra obtained after separation by HPLC, and effects of these components on the formation of the starch−iodine complex and pancreatin-induced digestion of starch were also studied (see below).

Effects of Fatty Acids. Fatty acids did not significantly affect the formation of the starch−iodine complex in control starch. Therefore, extracted starch was used to study the effects of fatty acids on the starch−iodine complex. Peaks of ΔA spectra between the presence and the absence of myristic, palmitic, and stearic acids were observed at 592, 600, and 570 nm, respectively (**Figure 6**, upper panel). Peaks of ΔA spectra were observed at 608, 620, and 654 nm for oleic, linoleic, and linolenic acids, respectively (data not shown). The peaks of ΔA indicate the suppression of the amylose−iodine complex formation by the fatty acids. The formation of amylose−lipid and −fatty acid complexes (*7*−*11*, *34*−*39*) and the inhibition of the starch−iodine complex formation by fatty acids (*40*) have been reported.

Figure 6 (lower panel) shows inhibition of pancreatin-induced starch digestion by myristic, palmitic, and stearic acids. The peak of ΔA spectrum in the absence of fatty acid was observed at 554 nm, whereas the peak in the presence of a fatty acid was observed at 536 nm. The fatty acid-dependent shift of the peak indicates that fatty acids inhibited the digestion of amylose. The $\Delta\Delta A$ spectrum (peak, approximately 680 nm) (inset) of trace

"without fatty acids" minus trace C in ΔA spectra indicates the preferential inhibition of amylose digestion by stearic acid. Such inhibition was also observed when other fatty acids were used. It has been reported that lipids preferentially inhibit amylase-catalyzed digestion of amylose (*7*−*11*). The results in **Figures 5** and **6** indicate that fatty acids in MP extract could contribute to the suppression of the amylose−iodine complex formation and the inhibition of pancreatin-induced digestion of amylose. Palmitic, oleic, linoleic, and linolenic acids have been reported to be contained in buckwheat flour (*41*).

Effects of Component X. No significant contaminants were detected in component X isolated in this study when analyzed by HPLC, and the isolate had *A* peaks at 204 and 267 nm with a shoulder at 300 nm, suggesting that component X was acylated if the component was proanthocyanidins or flavan-3-ols (*12*). The mass spectrum of component X showed mass numbers (*m*/*z*) of 469.65 $[M - H]^-$, 529.60 $[M - H +$ acetic acid$]^-$, and 940.15 $[2M - H]^-$. We estimated that this component was epicatechin-*O*-(3,4-dimethyl)-gallate (molecular weight, 470.42) (**Figure 7**). The estimation was supported by the detection of a component of mass number (*m*/*z*) of 271.4, which could be formed by dissociation of the dimethylgallate moiety from the mother molecule (*12*). The presence of epicatechin-*O*-(3,4-dimethyl)-gallate in buckwheat flour has been reported (*12*, *13*). The concentration of epicatechin-dimethylgallate was estimated using the molar extinction coefficient of epicatechin-*O*-gallate at 280 nm (ε = 12600 M^{-1} cm^{-1}) (*42*). The yield of epicatechin-dimethylgallate was not determined in this study. It has been reported that the content of epicatechin-*O*-(3,4-dimethyl)-gallate is 8.8 mg (18.7 μmol)/100 g of buckwheat groats (*13*).

Epicatechin-dimethylgallate did not affect *A* spectrum of an iodine solution in the wavelength range from 500 to 900 nm (data not shown) but suppressed the formation of the starch−iodine complex in extracted starch, and the suppression increased with the increase in the concentration of epicatechin-dimethylgallate (**Figure 8**, upper panel). ΔA spectra between the presence and the absence of epicatechin-dimethylgallate were different from the ΔA spectra observed using fatty acids (**Figure 6**, upper panel) but similar to the ΔA spectra observed in the presence of a large amount of MP extract (**Figure 5**, upper panel). Taking the results in **Figures 5** and **6** into account, the result in **Figure 8** indicates that epicatechin-dimethylgallate inhibited the formation of not only the amylose−iodine complex but also the amylopectin−iodine complex.

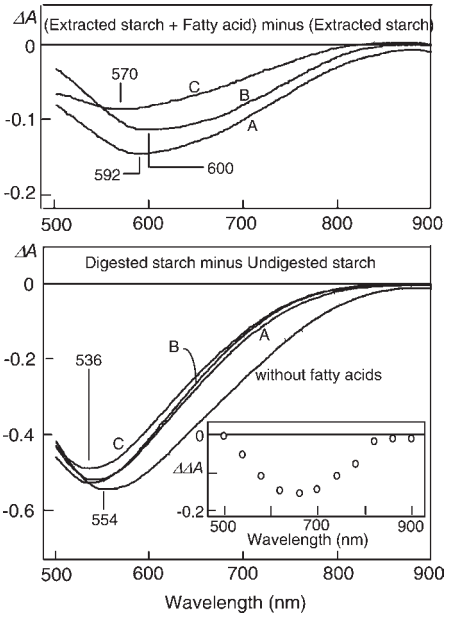

Figure 6. Interactions between extracted starch and fatty acids. Top panel, ΔA spectra between the presence and the absence of fatty acids. Concentration of fatty acids, 0.2 mM. Bottom panel, ΔA spectra before and after pancreatin treatment (2 min). Concentration of fatty acids, 0.5 mM. A, myristic acid; B, palmitic acid; and C, stearic acid. Inset: $\Delta\Delta A$ spectrum of trace "without fatty acids" minus trace C.

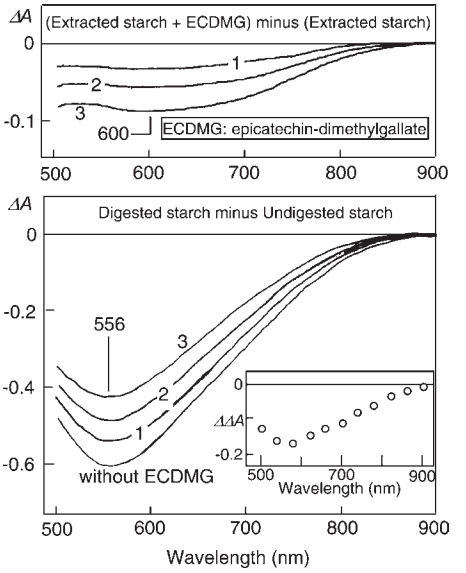

Figure 8. Interaction between epicatechin-dimethylgallate and extracted starch. Top panel, ΔA spectra between the presence and the absence of epicatechin-dimethylgallate. Bottom panel, ΔA spectra before and after pancreatin treatment (2 min). Trace 1, 7.6 μM; trace 2, 15 μM; and trace 3, 31 μM epicatechin-dimethylgallate. Inset: $\Delta\Delta A$ spectrum of trace "without epicatechin-dimethylgallate" minus trace 3. ECDMG, epicatechin-dimethylgallate.

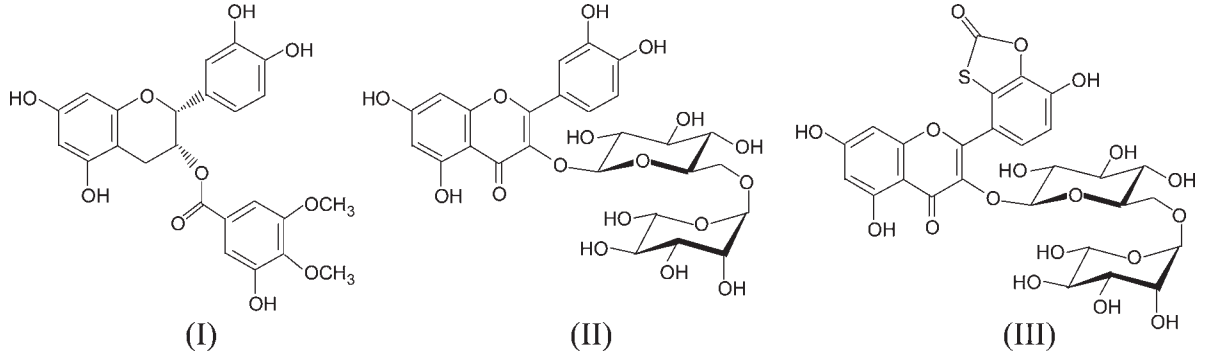

Figure 7. Structures of epicatechin-*O*-(4,5-dimethyl)-gallate (**I**), rutin (**II**), and an oxathiolone derivative of rutin (**III**).

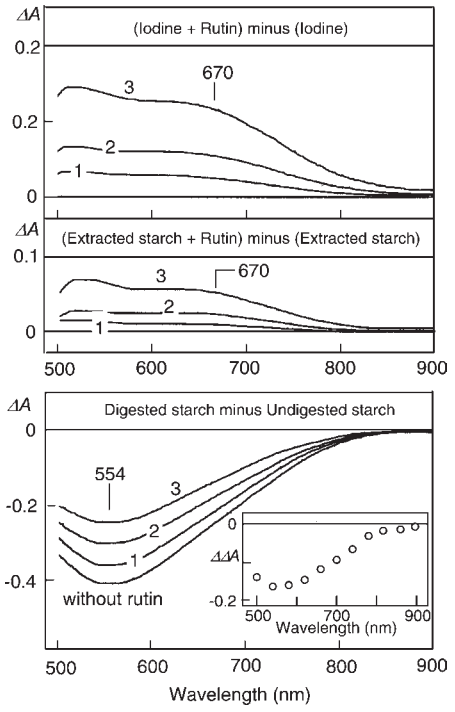

Figure 9. Interactions among rutin, iodine, and extracted starch. Top panel, ΔA spectra between the presence and the absence of rutin in an iodine solution. Middle panel, ΔA spectra between the presence and the absence of rutin in extracted starch. Bottom panel, effects of rutin on pancreatin-induced digestion of extracted starch. Digestion time, 2 min. Trace 1, 150 μM; trace 2, 300 μM; and trace 3, 600 μM rutin. Inset: $\Delta\Delta A$ spectrum of trace "without rutin" minus trace 3.

Difference spectra of the starch−iodine complex after and before the pancreatin-induced digestion indicate that epicatechin-dimethylgallate inhibited the digestion of extracted starch and the inhibition increased with the increase in concentration of epicatechin-dimethylgallate (**Figure 8**, lower panel). The peak of $\Delta\Delta A$ spectrum (590 nm) (inset) was shorter than the peak of $\Delta\Delta A$ spectrum (680 nm), which was obtained by trace "without fatty acids" minus trace C in **Figure 6** (lower panel), and $\Delta\Delta A$ at 500 nm relative to $\Delta\Delta A$ at 590 nm in **Figure 8** was much larger than $\Delta\Delta A$ at 500 nm relative to $\Delta\Delta A$ at 680 nm in **Figure 6**. Because amylose digestion was mainly inhibited in **Figure 6**, the result in **Figure 8** indicates that epicatechin-dimethylgallate inhibited pancreatin-induced digestion of not only amylose but also amylopectin.

Effects of Rutin and Quercetin. Fatty acids and epicatechin-dimethylgallate did not affect A spectrum of iodine in a buffer solution, but rutin (**Figure 7**), which was contained in buckwheat flour in large amounts [about 22 mg (36 μmol)/100 g of flour] (*43*), affected A spectrum of an iodine solution. ΔA spectra between the presence and the absence of rutin showed rutin-induced A increase, and the ΔA increased nearly linearly as a function of rutin concentration (**Figure 9**, upper panel). No changes in A spectra were observed (i) when rutin was mixed with iodine in 50 mM KCl−HCl (pH 2.0), (ii) when rutin was added to 18 mM KI solution in 50 mM KCl−HCl (pH 2.0) or 100 mM sodium phosphate (pH 7.1), and (iii) when an oxathiolone derivative of rutin [5,7-dihydroxy-2-(7-hydroxy-2-oxobenzo[*d*][1,3]oxathiol-4-yl)-4*H*-chromen-4-one 3-*O*-β-rutinoside] (150 μM) (**Figure 7**), which was prepared as reported previously (*44*), was mixed with iodine in the above buffer solutions with pH values of 2.0 and 6.8. These results suggest that rutin-induced changes in A spectra of the iodine solution (pH 6.8) were due to the formation of the rutin−iodine complex and that the catechol moiety of rutin contributed to the formation of the complex because no changes in A spectrum were observed when its catechol moiety was transformed to the benzoxathiolone moiety. In addition, the results suggest that an anion form of rutin (pK_a = 7.1) (*45*) contributed to the rutin−iodine complex.

Rutin also formed a rutin−iodine complex in the presence of starch, but the rutin-induced A increase was smaller in the presence than the absence of starch (**Figure 9**, middle panel). The result suggests that rutin interfered in the formation of the starch−iodine complex. If rutin did not interfere in the formation of starch−iodine complexes, ΔA spectra observed in the presence of starch should be similar to those observed in the absence of starch. The spectrum obtained by subtraction of spectrum 3 in the middle panel from spectrum 3 in the upper panel had peaks at approximately 540 and 660 nm (data not shown), suggesting that rutin inhibited the formation of not only the amylose−iodine complex but also the amylopectin−iodine complex.

Figure 9 (lower panel) shows that rutin inhibited pancreatin-induced digestion of starch and that the inhibition increased with the increase in rutin concentration. $\Delta\Delta A$ spectrum (inset) of trace "without rutin" minus trace 3 in ΔA spectra indicates that rutin inhibited the digestion of not only amylose but also amylopectin. It has been reported that rutin binds to starch during decoction of Chinese medicines and that the binding results in inhibition of amylase-catalyzed starch digestion (*46*). The oxathiolone derivative of rutin (150 μM) hardly inhibited pancreatin-induced digestion of extracted starch as well as the formation of the starch−iodine complex (data not shown).

Effects of quercetin on the formation of the starch−iodine complex were studied. Quercetin formed a reddish purple quercetin−iodine complex in an iodine solution (pH 6.8), and the A peak was observed around 520 nm, but no color changes were observed by mixing iodine with an oxathiolone derivative of quercetin [m/z 359.3 $[M - H]^-$; λ_{max} = 252 and 371 nm in methanol/25 mM KH_2PO_4 (2:1, v/v)], which was prepared by acid hydrolysis of the oxathiolone derivative of rutin. This result indicates an important function of the catechol moiety of quercetin in the formation of the quercetin−iodine complex. Quercetin also suppressed the formation of the starch−iodine complex as rutin (data not shown).

Pancreatin-induced digestion of extracted starch was inhibited by approximately 40% by 165 μM quercetin. $\Delta\Delta A$ spectrum between the presence and the absence of 165 μM quercetin was similar to that between the presence and the absence of rutin (cf. **Figure 9**), suggesting that quercetin inhibited the digestion of both amylose and amylopectin. The oxathiolone derivative of quercetin (139 μM) did not affect the formation of the starch−iodine complex but inhibited pancreatin-induced digestion of extracted starch by 5−15%, indicating that the oxathiolone derivative was not such an effective inhibitor.

Binding of Epicatechin-Dimethylgallate and Rutin to Amylose and Amylopectin. The above results indicate that fatty acids suppressed the formation of the amylose−iodine complex, whereas epicatechin-dimethylgallate and rutin suppressed the formation of not only the amylose−iodine complex but also the amylopectin−iodine complex. To confirm the latter, we studied effects of these phenolic compounds on the formation of the amylopectin−iodine complex (**Figure 10**). The A spectrum of an iodine solution was not affected by epicatechin-dimethylgallate as described above (trace A in middle upper panel) but suppressed the formation of not only the amylose−iodine complex (trace B) but also the amylopectin−iodine complex (traces C-1 and C-2 in middle upper panel). Rutin also suppressed the formation of the amylopectin−iodine complex (trace C) as well as the amylose−iodine complex (trace B in middle lower panel). The suppression is clear if one takes $\Delta\Delta A$ spectra of

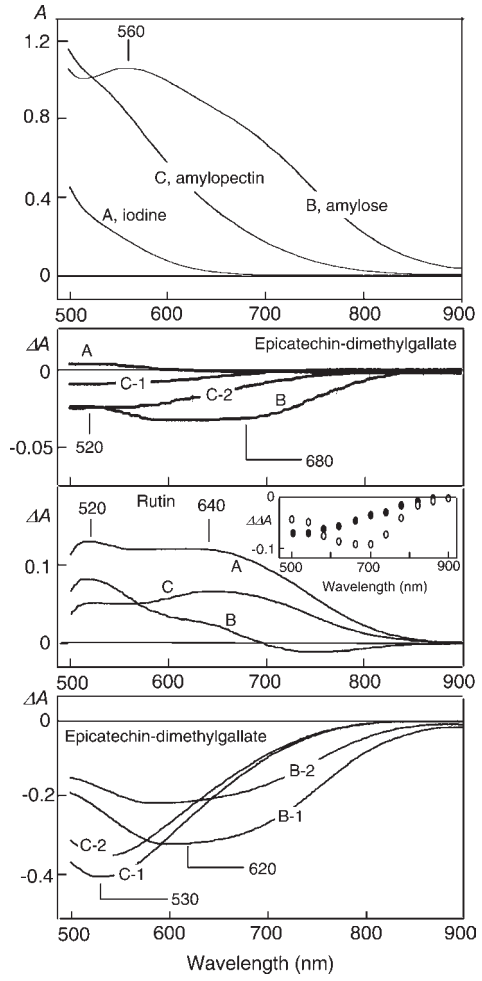

Figure 10. Interactions of epicatechin-dimethylgallate and rutin with amylose and amylopectin. Top panel, *A* spectra of starch−iodine complexes. A, 10 mM iodine; B, iodine/amylose (30 μg/mL); and C, iodine/amylopectin (20 μg/mL). Middle upper panel, ΔA spectra between the presence and the absence of epicatechin-dimethylgallate. A, iodine ± 31 μM epicatechin-dimethylgallate; B, iodine/amylose ± 15 μM epicatechin-dimethylgallate; C-1, iodine/amylopectin ± 15 μM epicatechin-dimethylgallate; and C-2, iodine/amylopectin ± 31 μM epicatechin-dimethylgallate. Middle lower panel, ΔA spectra between the presence and the absence of rutin. A, iodine ± 600 μM rutin; B, iodine/amylose ± 600 μM rutin; and C, iodine/amylopectin ± 600 μM rutin. Inset: $\Delta\Delta A$ spectrum. ○, B minus A; ●, C minus A. Bottom panel, epicatechin-dimethylgallate-dependent inhibition of pancreatin-induced digestion of amylose and amylopectin. B-1 and C-1, without epicatechin-dimethylgallate; B-2 and C-2, with 31 μM epicatechin-dimethylgallate. Digestion time, 1 min.

trace B minus trace A (○) and trace C minus trace A (●) (inset). The result in **Figure 10** (middle panels) coincides with the conclusion induced from **Figures 8** and **9** that epicatechin-dimethylgallate and rutin bound to both amylose and amylopectin in extracted starch.

Effects of epicatechin-dimethylgallate on pancreatin-induced digestion of amylose and amylopectin were studied (**Figure 10**, lower panel). Epicatechin-dimethylgallate (31 μM) inhibited the digestion of amylose (compare traces B-1 and B-2) more effectively than that of amylopectin (compare traces C-1 and C-2). The effects of 600 μM rutin on the pancreatin-induced digestion of amylose and amylopectin were similar to those of 31 μM epicatechin-dimethylgallate (data not shown). These results indicate that epicatechin-dimethylgallate and rutin could inhibit the digestion of amylose more effectively than that of amylopectin, supporting the idea that epicatechin-dimethylgallate and rutin inhibited pancreatin-induced digestion of amylose and amylopectin in extracted starch. We could deduce from the effects of epicatechin-dimethylgallate on the starch−iodine complex formation and the starch digestion in **Figures 8** and **10** that proanthocyanidins contained in buckwheat flour might also have functions similar to those of epicatechin-dimethylgallate.

Epicatechin-dimethylgallate inhibited the formation of the starch−iodine complex and pancreatin-induced digestion of starch at dozens of micromolar, whereas rutin inhibited them at several hundred micromolar. The difference might be attributed to the presence or absence of rutinose or the difference in hydrophobicity. This idea is supported by the result that 165 μM quercetin and 150 μM rutin inhibited pancreatin-induced digestion of starch by approximately 40 and 10%, respectively.

The present study indicated that buckwheat starch could interact with fatty acids, rutin, and epicatechin-dimethylgallate, which were contained in buckwheat flour. In these components, fatty acids seemed to be more effective in the inhibition in respect to amylose digestion, because the addition of small amounts of MP extract preferentially suppressed the digestion amylose (**Figure 5**). According to refs *34*−*39*, fatty acids may bind to the hydrophobic helical structure of amylose inhibiting its digestion. Epicatechin-dimethylgallate and rutin bound to amylose and amylopectin. Amylopectin also has a hydrophobic helical structure in the molecule, but the length of the helical structure of amylopectin is shorter than that of amylose. Smaller sizes of hydrophobic regions of epicatechin-dimethylgallate and rutin than the fatty acids may make these phenolic compounds possible to bind to hydrophobic helical structure of not only amylose but also amylopectin. The binding resulted in the inhibition of amylase-catalyzed digestion of both amylose and amylopectin. It has been reported that phenolic compounds can inhibit amylase activity (*22*−*27*), but such inhibition seems not to occur in the present study, because MP extract inhibited pancreatin-induced digestion of extracted starch but not control starch. The different effects of epicatechin-dimethylgallate and rutin on the digestion of amylose and amylopectin also support that the inhibitory effects were not due to the inhibition of amylase activity in pancreatin. There are other possibilities for slower digestion of control starch than extracted starch. These are the formation of covalent bonds between phenolic compounds contained in buckwheat flour and buckwheat starch during boiling (*47*, *48*). The formation of the covalent bonds may result in the generation of stable radicals (*47*). Further studies are required from these points.

The oxathiolone derivative of rutin can be produced in the stomach by its oxidation in the presence of thiocyanate (*47*), and the rutin oxathiolone derivative may be transformed to the oxathiolone derivative of quercetin by hydrolysis in the intestine. These components seemed not to affect starch digestion in the intestine because their effects on the inhibition of amylase-catalyzed digestion of starch were much smaller than those of rutin and quercetin.

According to the above discussion, we can postulate that the smaller susceptibility of control buckwheat starch than extracted buckwheat starch to amylase (**Figure 3**) may be due to the binding of fatty acids, rutin, and proanthocyanidins including flavan-3-ols to buckwheat starch. Therefore, the ability of buckwheat flour to decrease the glycemic and insulin indexes of products made from wheat flour (*1*) could be attributed to the binding of fatty acids, rutin, and proanthocyanidins including flavan-3-ols, which were contained in buckwheat flour, to wheat flour starch.

ACKNOWLEDGMENT

We are grateful to Kitakyushu Life Science Center for the measurements of LC/MS.

LITERATURE CITED

(1) Skrabanja, V.; Elmståhl, H. G. M. L.; Kreft, I.; Björck, I. M. E. Nutritional properties of starch in buckwheat products: Studies in vitro and in vivo. *J. Agric. Food Chem.* **2001**, *49*, 490−496.

(2) Qin, P.; Wang, Q.; Shan, F.; Hou, Z.; Ren, G. Nutritional composition and flavonoids content of flour from different buckwheat cultivars. *Int. J. Food Sci. Technol.* **2010**, *45*, 951−958.

(3) Skrabanja, V.; Kreft, I. Resistance starch formation following autoclaving of buckwheat (*Fagopyrum esculentum* Moench) groats. An in vitro study. *J. Agric. Food Chem.* **1998**, *46*, 2020−2023.

(4) Christal, K.; Soral-Smietanal, M.; Lewandowicz, G. Buckwheat starch: Structure, functionality and enzyme in vitro susceptibility upon the roasting process. *Int. J. Food Sci. Nutr.* **2009**, *60*, 140−154.

(5) Qian, J.; Rayas-Duarte, P.; Grant, L. Partial characterization of buckwheat (*Fagopyrum esculentum* Moench) starch. *Cereal Chem.* **1998**, *75*, 365−373.

(6) Yoshimoto, Y.; Egashira, T.; Hashimoto, I.; Ohinata, H.; Takase, Y.; Takeda, Y. Molecular structure and some physicochemical properties of buckwheat starch. *Cereal Chem.* **2004**, *81*, 515−520.

(7) Seneviratne, H. D.; Biliaderis, C. G. Action of α-amylases on amylose-lipid complex superstructures. *J. Cereal Sci.* **1991**, *13*, 129−143.

(8) Crowe, T. C.; Seligman, S. A.; Copeland, L. Inhibition of enzymatic digestion of amylose by free fatty acids in vitro contributes to resistant starch formation. *J. Nutr.* **2000**, *130*, 2006−2008.

(9) Nebesny, E.; Rosicka, J.; Tkaczyk, M. Effect of enzymatic hydrolysis of wheat starch on amylose-lipid complexes stability. *Starch/Stärke* **2002**, *54*, 603−608.

(10) Holm, J.; Björck, I.; Ostrowska, S.; Eliasson, A.-C.; Asp, N.-G.; Larsson, K.; Lundquist, I. Digestibility of amylose-lipid complexes in-vitro and in-vivo. *Starch/Stärck* **2006**, *35*, 294−297.

(11) Kwasniewska-Karolak, I.; Nebesny, E.; Rosicka-Kaczmarek, J. Characterization of amylose-lipid complexes derived from different wheat varieties and their susceptibility to enzymatic hydrolysis. *Food Sci. Technol. Int.* **2008**, *14*, 29−37.

(12) Ölschläger, C.; Regos, I.; Zeller, F. J.; Treutter, D. Identification of galloylated propelargonidins and procyanidins in buckwheat grain and qunatification of rutin and flavanols from homostylous hybrids originating from *F. esculentum* × *F. homotropicum*. *Phytochemistry* **2008**, *69*, 1389−1397.

(13) Watanabe, M. Catechins as antioxidants from buckwheat (*Fagopyrum esculentum* Möench) groats. *J. Agric. Food Chem.* **1998**, *46*, 839−845.

(14) Davis, A. B.; Hoseney, R. C. Grain sorghum condensed tannins. 1. Isolation, estimation, and selective adsorption by starch. *Cereal Chem.* **1979**, *56*, 310−314.

(15) Cohen, R.; Orlova, Y.; Kovalev, M.; Ungar, Y.; Shimoni, E. Structural and functional properties of amylose complexes with genistein. *J. Agric. Food Chem.* **2008**, *56*, 4212−4218.

(16) Renard, C. M. G. C.; Baron, A.; Guyot, S.; Drilleau, J. F. Interactions between apple cell walls and native apple polyphenols: Quantification and some consequences. *Int. J. Biol. Macromol.* **2001**, *29*, 115−125.

(17) Le Bourvellec, C.; Bouchet, B.; Renard, C. M. Non-covalent interaction between procyanidins and apple cell wall material. Part III: Study on model polysaccharides. *Biochim. Biophys. Acta* **2005**, *1725*, 10−18.

(18) Cai, Y.; Gaffney, S. H.; Lilley, T. H.; Magnoloto, M.; Martin, R.; Spencer, C. M.; Haslam, E. Polyphenols interactions. Part 4. Model studies with caffeine and cyclodextrins. *J. Chem. Soc. Perkin Trans. 2* **1990**, 2197−2209.

(19) Fayad, N.; Marchal, L.; Billaud, C.; Nicolas, J. Comparison of beta-cyclodextrin effect on polyphenol oxidation catalyzed by purified polyphenol oxidase from different sources. *J. Agric. Food Chem.* **1997**, *45*, 2442−2446.

(20) Ishizu, T.; Kintsu, K.; Yamamoto, H. NMR study of the solution structures of the inclusion complexes of beta-cyclodextrin with (+)-catechin and (−)-epicatechin. *J. Phys. Chem. B* **1999**, *103*, 8992−8997.

(21) Kriz, Z.; Koca, J.; Imberty, A.; Charlot, A.; Auzely-Velty, R. Investigation of the complexation of (+)-catechin by beta-cyclodextrin by a combination of NMR, microcalorimetry and molecular modeling techniques. *Org. Biomol. Chem.* **2003**, *14*, 2590−2595.

(22) Kim, J.-S.; Kwon, C.-S.; Son, K. H. Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 2458−3461.

(23) Lee, Y. A.; Cho, E. J.; Tanaka, T.; Yokozawa, T. Inhibitory activities of proanthocyanidins from persimmon against oxidative stress and digestive enzymes related to diabetes. *J. Nutr. Sci. Vitaminol.* **2007**, *53*, 287−292.

(24) Li., Y.; Gao, F.; Gao, F.; Shan, F.; Bian, J.; Zhao, C. Study on the interaction between 3 flavonoid compounds and α-amylase by fluorescence spectroscopy and enzyme kinetics. *J. Food Sci.* **2009**, *74*, C199−C203.

(25) Kim, H.; Kim, J.-K.; Kang, L.; Jeong, K.; Jung, S. Docking and scoring of quercetin and quercetin glycosides against α-amylase receptor. *Bull. Korean Chem. Soc.* **2010**, *31*, 461−463.

(26) Narita, Y.; Inouye, K. Kinetic analysis and mechanism on the inhibition of chlorogenic acid and its components against porcine pancreas α-amylase isozymes I and II. *J. Agric. Food Chem.* **2009**, *57*, 9218−9225.

(27) He, Q.; Lv, Y.; Yao, K. Effects of tea polyphenols on the activities of α-amylase, pepsin, trypsin and lipase. *Food Chem.* **2006**, *101*, 1178−1182.

(28) Swanson, M. A. Studies on the structure of polysaccharides IV. Relation of the iodine color to the structure. *J. Biol. Chem.* **1948**, *172*, 825−837.

(29) Abe, T. The visible and ultraviolet absorption spectra of cellulose- and amylose-iodine complexes. *Bull. Chem. Soc. Jpn.* **1958**, *31*, 661−662.

(30) Yu, X.; Houtman, C.; Atalla, R. H. The complex of amylose and iodine. *Carbohydr. Res.* **1996**, *292*, 129−141.

(31) Konishi, Y.; Nojima, H.; Okuno, K.; Asaoka, M.; Fuwa, H. Characterization of starch granules form waxy, nonwaxy, and hybrid seeds of *Amaranthus hypochondriacus* L. *Agric. Biol. Chem.* **1985**, *49*, 1965−1971.

(32) Fukahori, N.; Yamashita, S.; Baba, K. Influence of cultivars of rice and molecular weight of amylose on the selected wavelength for the dual-wavelength method. *Bull. Fukuoka Agric. Res. Cent.* **1996**, *15*, 15−17.

(33) Lever, M. A new reaction for colorimetric determination of carbohydrates. *Anal. Biochem.* **1972**, *47*, 273−279.

(34) Taylor, T. C.; Sherman, R. T. Carbohydrate-fatty acid linkings in corn alpha amylose. *J. Am. Chem. Soc.* **1933**, *55*, 258−264.

(35) Takeo, K.; Tokumura, A.; Kuge, T. Complexes of starch and its related materials with organic compounds. Part X. X-ray diffraction of amylose-fatty acid complexes. *Starch/Stärke* **1973**, *25*, 357−362.

(36) Godet, M. C.; Bouchet, B.; Colonna, P.; Gallant, A. Crystaline amylose-fatty acid complexes: morphology and crystal thickness. *J. Food Sci.* **1996**, *61*, 1196−1201.

(37) Akuzawa, S.; Sawayama, S.; Kuwabata, A. Thermal properties of corn amylose incorporating or with added free fatty acid. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 487−490.

(38) Kaur, K.; Singh, N. Amylose-lipid complex formation during cooking of rice flour. *Food Chem.* **2000**, *71*, 511−517.

(39) Shogren, R. L.; Fanta, G.; Felker, F. C. X-ray diffration study of crystal transformations in spherulitic amylose/lipid complexes from jet-cooked starch. *Carbohydr. Res.* **2006**, *64*, 444−451.

(40) Bhatnagar, S.; Hanna, M. A. Amylose-lipid complex formation during single-screw extrusion of various corn starch. *Cereal Chem.* **1994**, *71*, 582−587.

(41) Bonafaccia, G.; Marocchini, M.; Kreft, I. Composition and technological properties of flour and bran from common and tartary buckwheat. Trace elements in flour and bran from common and tartary buckwheat. *Food Chem.* **2003**, *80*, 9−15.

(42) Koerner, J. L.; Hsu, V. L.; Lee, J.; Kennedy, J. A. Determination of proanthocyanidin A2 content in phenolic polymer isolates by

reverse-phase high-performance liquid chromatography. *J. Chromatogr. A* **2009**, *1216*, 1403−1409.
(43) Kreft, I.; Fabjan, N.; Yasumoto, K. Rutin content in buckwheat (*Fagopyrum esculentum* Moench) food materials and products. *Food Chem.* **2006**, *98*, 508−512.
(44) Takahama, U.; Tanaka, M.; Hirota, S.; Yamauchi, R. Formation of oxathiolone compound from rutin in acidic mixture of saliva and buckwheat dough: Possibility of its occurrence in the stomach. *Food Chem.* **2009**, *116*, 214−219.
(45) Arshad, N.; Janjua, N. K.; Ahmed, S.; Khan, A. Y.; Skibsted, L. H. Electrochemical investigations of antioxidant interactions with radical anion and dianion of 1,3-dinitrobenzen. *Electrochim. Acta* **2009**, *54*, 6184−6189.
(46) Takaishi, K.; Torii, Y. Studies on the decoction of Chinese medicines. I. On the interaction of some chemicals with starch in aqueous solution. *Yakugaku Zasshi* **1969**, *89*, 538−543.
(47) Takahama, U.; Tanaka, M.; Hirota, S. Proanthocyanidins in buckwheat flour can reduce salivary nitrite to nitric oxide in the stomach. *Plant Foods Hum. Nutr.* **2010**, *65*, 1−7.
(48) Beart, J. E.; Lilley, T. H.; Haslam, E. Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalyzed decomposition; observations on some polymeric proanthocyanidins. *J. Chem. Soc. Perkin Trans. 2* **1985**, 1439−1443.

Received for review February 24, 2010. Revised manuscript received September 23, 2010. Accepted September 23, 2010.