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Formation of an oxathiolone compound from rutin in acidic mixture of saliva and buckwheat dough: Possibility of its occurrence in the stomach

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ABSTRACT

Buckwheat is used for various foods in the world. The objective of the present study is the determination of the chemical structure of a product formed in the acidic mixture of buckwheat dough and saliva that contains both nitrite and thiocyanate. In the mixture, 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, was found. This compound seemed to be formed by the hydrolysis of a 2′-thiocyanate derivative of rutin, which was produced by the reaction of thiocyanate with the *o*-quinone generated by nitrous acid-dependent oxidation of rutin. Importance of the formation of the oxathiolone compound is discussed in relation to scavenging of the toxic quinone in the stomach.

1. Introduction

The flour of buckwheat is used for various foods in the world. For example, buckwheat noodles are eaten in Japan, Korea and Northern Italy, buckwheat pancakes are eaten in Russia and France, and buckwheat groats are consumed in western Asia and Eastern Europe. In the some countries of Japan, buckwheat flour is kneaded with hot water preparing dough, and the dough is tasted as a snack. Buckwheat seeds contain antioxidants like rutin and proanthocyanidins and the flour also contains antioxidants (Kreft, Fabjan, & Yasumoto, 2006; Kreft, Knapp, & Kreft, 1999; Ölschläger, Regos, Zeller, & Treutter, 2008; Watanabe, Ohshita, & Tsushida, 1997). The content of rutin in the flour is estimated to be 12.7–21.8 mg/100 g (Danila, Kotani, Hakamata, & Kusu, 2007; Kreft et al., 2006). On the other hand, buckwheat leaves, which also contain rutin and proanthocyanidins, are often used as a herbal tea. It has been reported that the herbal tea can increase the microcirculation in people with chronic venous insufficiency (Ihme et al., 1996).

When foods and beverages prepared from buckwheat are taken, they are mixed with saliva and then swallowed into the stomach. In the stomach, the mixture is acidified by gastric juice. Saliva contains nitrite (p*K*a = 3.3) and SCN^-. The nitrite in saliva is transformed to nitrous acid, which can lead to nitration, nitrosation and oxidation, in the stomach. It has been reported that in addition to ascorbic acid, *o*-diphenols like quercetin, caffeic acid and chlorogenic acid are able to reduce nitrous acid to nitric oxide (NO) (Gago, Lundberg, Barbosa, & Laranjinha, 2007; Peri et al., 2005; Takahama, Oniki, & Hirota, 2002; Takahama, Tanaka, Oniki, Hirota, & Yamauchi, 2007). Melanoidins of coffee are also able to reduce nitrous acid to NO (Takahama & Hirota, 2008). When chlorogenic acid is oxidised to its quinone form by nitrous acid, the quinone form reacts with SCN^- producing 2-thiocyanatechlorogenic acid that is hydrolysed to the oxathiolone derivative and NH_3 (Takahama et al., 2007). The production of the oxathiolone compound is possible in the stomach after drinking coffee (Takahama et al., 2007). The aim of the present study is to show the formation of an oxathiolone compound from rutin (Fig. 1) in the acidic mixture of saliva and buckwheat dough, simulating the mixture of buckwheat dough, saliva and gastric juice in the stomach. The significance of the formation of oxathiolone compounds is discussed in relation to the scavenging of toxic quinones produced by nitrous acid-dependent oxidation of diphenols in the stomach.

2. Materials and methods

2.1. Reagents

Rutin and Griess-Romijn reagent for nitrite were obtained from Wako Pure Chem. Ind. (Osaka, Japan). Flour of buckwheat (*Fagopyrum*

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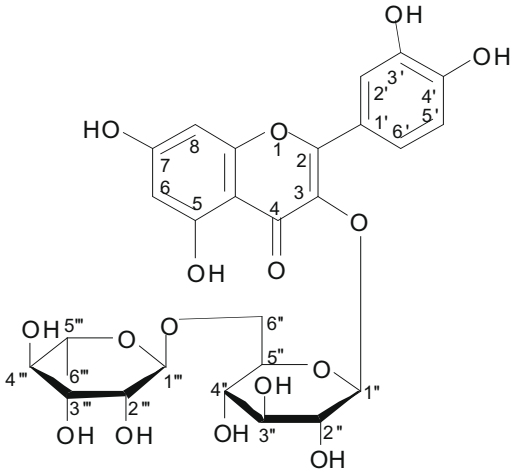

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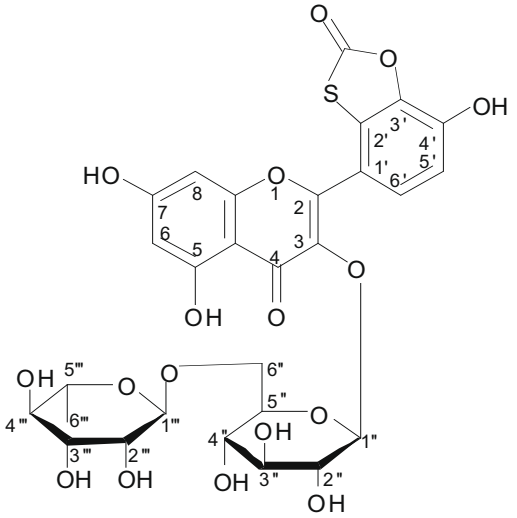

(2)

Fig. 1. Structures of rutin (**1**) and component *Y* (**2**) isolated in this study.

esculentum Moench) was obtained from a local market. Rutin content of the flour was about 0.3 mg/g of flour. The content was estimated by HPLC (see below) after extracting the flour (0.1 g) with 5 ml of methanol and the value was close to the value reported previously (Danila et al., 2007; Kreft et al., 2006).

2.2. Preparation of reaction mixtures

The decrease in the concentration of rutin and the formation of its reaction products in the acidic mixture of saliva and buckwheat flour were studied as following. Buckwheat flour (5 g) was kneaded with 3 ml of hot water to prepare dough. The dough (1 g) was mixed with saliva in the oral cavity by chewing for 1 min. The pH of the mixture was adjusted to 2 by the addition of a small amount of 2 M HCl, and then centrifuged at 1500*g* for 2 min. The acidic supernatant was kept at room temperature (about 27 °C) and 10 μl of the supernatant was analysed by HPLC every 20 min. Prior to the analysis, samples were passed through a cellulose acetate filter (0.45 μm, Advantec, Tokyo, Japan). When required, 10 μl of 100 mM $NaNO_2$ was added to 1 ml of the supernatant immediately after the centrifugation to enhance the decrease in the concentration of rutin and the formation of its reaction products.

The decrease in the concentration of rutin and the formation of its reaction products were also studied using buckwheat flour (0.1 g) suspended in 5 ml of 50 mM KCl–HCl buffer (pH 1.76) in the presence of 1 mM $NaNO_2$ and 1 mM NaSCN. The final pH was about 2. The suspension was centrifugation at 1500*g* for 2 min, and then the supernatant was incubated for defined periods at room temperature to be analysed by HPLC after passing through a cellulose acetate filter (see above).

Furthermore, the decrease in the concentration of rutin and the formation of its reaction products were measured in acidic buffer solution. The reaction mixture contained 1 mM rutin in 50 mM KCl–HCl buffer (pH 2.0) in the presence of 1 mM $NaNO_2$ and 1 mM NaSCN. After incubation for defined periods, 10 μl of the reaction mixture was directly applied to a HPLC column to quantify rutin and the reaction products.

2.3. HPLC

HPLC was performed using a LC-6A pump and a Shim-pack CLC-ODS column (15 cm × 6 mm i.d.) (Shimadzu, Kyoto, Japan). The mobile phase was a mixture of methanol and 25 mM KH_2PO_4 (pH 4.5) (1:1, v/v) and the flow rate was 1 ml/min. Rutin and its reaction products separated by the column were detected at 370 nm using a spectrophotometric detector with a photodiode array (SPD-M10Avp, Shimadzu). The concentration of each component separated was estimated from the area under the peak. The content of rutin in buckwheat flour was estimated using 1 mM rutin as a standard.

2.4. Spectrophotometric measurements

Nitrite-induced changes in absorption spectra were monitored using an UV/Vis spectrophotometer (UV-260, Shimadzu) in a reaction mixture (1 ml) that contained 50 μM rutin and 0.2 mM $NaNO_2$ in 50 mM KCl–HCl buffer (pH 2.0) in the presence and absence of 1 mM NaSCN. The path length of the measuring beam was 4 mm and reactions were started by the addition of 2 μl of 100 mM $NaNO_2$.

2.5. Isolation of a reaction product formed in the mixture of rutin, nitrite and SCN

Rutin and NaSCN were dissolved in dimethylsulfoxide and water at concentrations of 100 mM and 1 M, respectively. Four ml of the rutin solution (244 mg; final concentration, about 2 mM) and 2 ml of the NaSCN solution (final concentration, about 10 mM) were added to 200 ml of 50 mM KCl–HCl buffer (pH 2.0), and then 6 ml of 100 mM $NaNO_2$ was added slowly to the mixture (final concentration, about 3 mM). After incubation for 1 h at room temperature (about 27 °C), water was evaporated with a rotary evaporator. The residue was dissolved in 20 ml of methanol and methanol insoluble components were removed by centrifugation. Methanol of the methanol solution was removed with a rotary evaporator, and methanol (20 ml) was added again to the residue. After removing methanol insoluble components by centrifugation, the methanol solution was concentrated to about 1 ml, and then 8 ml of water was added to the concentrate. Pale yellow precipitate was formed when the water-added concentrate was kept at 4 °C for one night. The precipitate was washed twice with water and dried *in vacuo* over NaOH. The yield was 44 mg. HPLC analysis showed that the precipitate was constituted of component *Y* (see below).

2.6. Nuclear magnetic resonance (NMR) spectra, mass spectra and elemental analysis

^{1}H and ^{13}C NMR spectra were recorded with a ECX-400P FT-NMR spectrometer (JEOL, Tokyo, Japan) with dimethylsulfoxide-d_6 (DMSO-d_6) as the solvent and tetramethylsilane as the internal standard. ^{1}H NMR was performed at 399.78 MHz, and the ^{1}H–^{1}H chemical shift correlated (COSY) technique was employed to assign ^{1}H shifts and couplings. ^{13}C NMR was at 100.53 MHz with proton decoupling. Heteronuclear multiple-bond correlation (HMBC) and heteronuclear multiple-quantum coherence (HMQC) techniques were used to assign correlations between ^{1}H and ^{13}C signals.

Electrospray ionisation mass spectrum (ESI-MS) was obtained with a LC-MS QP8000α quadrupole mass spectrometer (Shimadzu). Sample was delivered into the ion source using 60% methanol containing 0.2% formic acid at 0.15 ml/min. Ionisation was performed with an ESI probe voltage of +4.5 kV for the positive-ion mode or −3.5 kV for the negative-ion mode. Atmosphere-pressure chemical ionisation mass spectrum (APCI-MS) was obtained with the mass spectrometer and the mobile phase described above. Ionisation was performed with an APCI probe voltage of +4.5 kV for the positive-ion mode or −3.0 kV for the negative-ion mode. Nebulizing gas (nitrogen) was delivered at flow rates of 4.5 and 2.5 l/min for ESI-MS and APCI-MS, respectively, and the curved desolvation line (CDL) was maintained at 250 °C during the measurement of mass spectra by the two mass spectrometers. Elemental analysis was performed using a CHN Corder MT-6 apparatus (Yanako, Kyoto, Japan).

3. Results and discussion

3.1. Characterisation of components formed during incubation of saliva and buckwheat dough

Saliva and buckwheat dough were mixed in the oral cavity by chewing and the pH of the mixture was decreased to 2 by adding HCl. Immediately after centrifugation of the acidified mixture, the supernatant was analysed by HPLC. Typical HPLC profiles are shown in Fig. 2 (upper panel, A-1). Rutin, which was identified by comparing its retention time and the absorption spectrum with the standard compound, was detected at a retention time of about 6 min. The concentration of rutin decreased to about 80% of the initial concentration after the incubation for 60 min, but no reaction products of rutin were detected (A-2). Nitrite (final concentration, 1 mM) was added to the supernatant obtained by centrifugation of the acidic mixture of saliva and buckwheat dough and the concentration of rutin was measured after the incubation for 60 min. The concentration was less than 5% of the initial concentration (B-2). Components *X* (retention time, about 13 min) and *Y* (retention time, about 18 min) were generated accompanying the decrease in concentration of rutin. Typical time courses of the changes in concentrations of rutin and components *X* and *Y* are shown in Fig. 2 (lower panel). The time courses show that the concentration of component *X* increased at first and then the concentration of component *Y* increased, suggesting that rutin was transformed to component *Y* via component *X*.

The concentration of nitrite in the mixture of saliva and dough in Fig. 2 was about 0.1 mM when determined using Griess-Romijn reagent (Takahama, Hirota, Yamamoto, & Oniki, 2003). Catechins (Danila et al., 2007) and proanthocyanidins (Kreft et al., 1999; Ölschläger et al., 2008; Watanabe et al., 1997) in buckwheat flour were rapidly oxidised by nitrite under acidic conditions (data not shown). In the mixture of saliva and dough, catechin and pronathocyanidins should also be contained. Therefore, in the presence of low concentrations of nitrite, even when rutin was oxidised, the

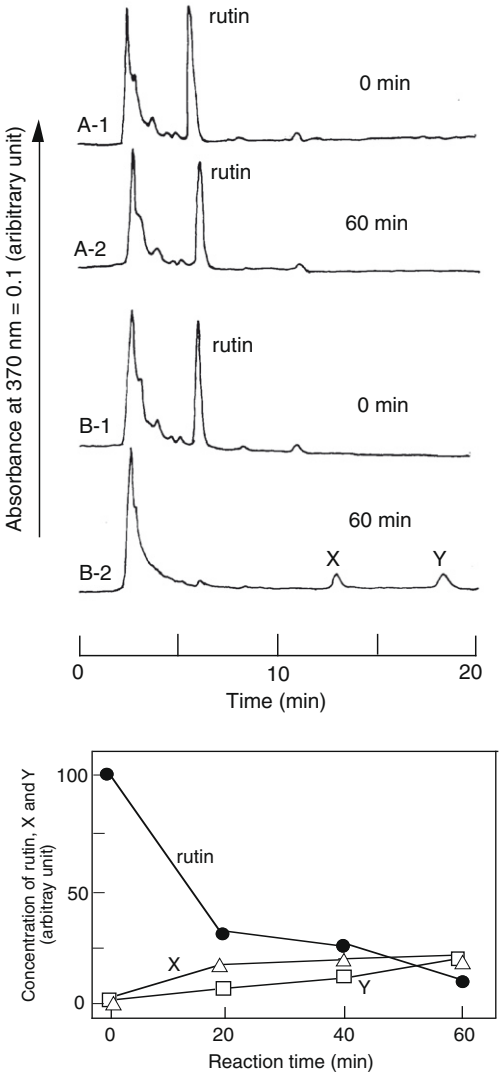

Fig. 2. Changes in concentration of rutin in the acidic mixture of buckwheat dough and saliva. Upper panel: HPLC profiles. The acidic supernatant of the mixture of saliva and dough was incubated for 0 and 60 min in the presence (B) and absence (A) of 1 mM $NaNO_2$, respectively. *X* and *Y* represent components formed during the incubation. Lower panel: Time courses of decrease in concentration of rutin and formation of components *X* and *Y* in the presence of 1 mM $NaNO_2$. The concentration of rutin is expressed relative to the initial concentration and the concentrations of components *X* and *Y* are expressed as arbitrary unit using areas under the peaks of HPLC. ●, rutin; △, component *X*; □, component *Y*.

oxidation intermediates might react with the oxidation intermediates of catechin and proanthocyanidins, resulting in the failure of the formation of components *X* and *Y*. The formation of components *X* and *Y* by the addition of 1 mM nitrite suggests that the components may be formed in the stomach after eating dough of buckwheat flour if the concentration of nitrite in saliva is high. It has been reported that the concentration of nitrite in saliva increases to about 1 mM after the ingestion of nitrate rich foods (Pannala et al., 2003).

To understand more exactly how components *X* and *Y* were formed in the acidic mixture of saliva and dough, we suspended the flour in 50 mM KCl–HCl (pH 1.76) and then measured the formation of components *X* and *Y* in the presence of 1 mM nitrite and 1 mM SCN^- (Fig. 3). During the incubation of the acidic suspension, the concentration of rutin decreased with a half-life of about 10 min and components *X* and *Y* were formed. The changes in concentrations of rutin and components *X* and *Y* support the idea that

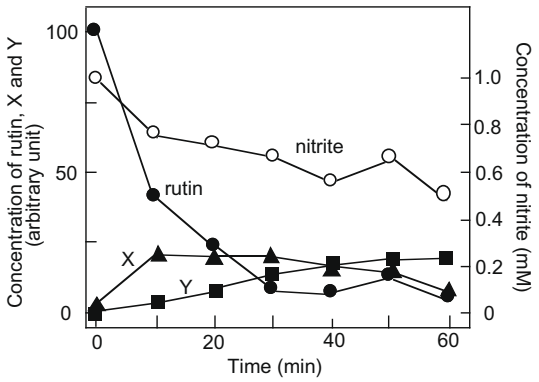

Fig. 3. Time courses of decrease in concentration of rutin and formation of components *X* and *Y* in acidic suspension of buckwheat flour. The reaction mixture (5 ml) contained 0.1 g of buckwheat flour, 1 mM $NaNO_2$ and 1 mM NaSCN in 5 ml of 50 mM KCl–HCl buffer (pH 1.76). The final pH was about 2. The concentrations of rutin and components *X* and *Y* are expressed as in Fig. 2. ●, rutin; ▲, component *X*; ■, component *Y*. The decrease in concentration of nitrite was also included in the figure (○).

rutin was transformed to component *Y* via component *X* (Fig. 3). Accompanying the decrease in the concentration of rutin, nitrite was consumed. In the absence of SCN^-, nitrite (1 mM) decreased the concentration of rutin with a half-life of about 10 min, but no formation of components *X* and *Y* was observed (not shown). This result indicates that SCN^- was essential for the formation of components *X* and *Y*. To confirm further the transformation of rutin to components *X* and *Y* in the acidic mixture of saliva and dough, rutin was incubated in the presence of 1 mM nitrite and 1 mM SCN^- in 50 mM KCl–HCl (pH 2.0). As expected, the two components were produced during the incubation, and time courses of the formation clearly showed that rutin was transformed to component *Y* via component *X* (Fig. 4). Absorption spectra of components *X* and *Y* separated by HPLC are shown in Fig. 5, and the spectra resembled each other.

We recorded nitrite-induced changes in the absorption spectra of rutin in 50 mM KCl–HCl (pH 2) in the presence and absence of SCN^- (Fig. 6). In the absence of SCN^-, the absorbance increased

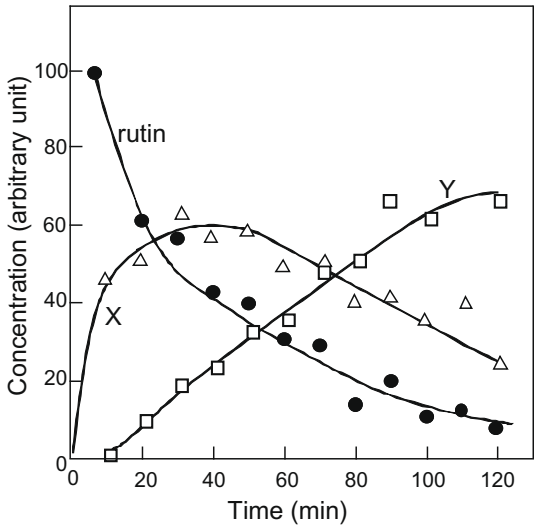

Fig. 4. Formation of components *X* and *Y* by a rutin/nitrite/SCN^- system. Time courses of formation of components *X* and *Y*. The reaction mixture (1 ml) contained 1 mM rutin, 1 mM $NaNO_2$ and 1 mM NaSCN in 50 mM KCl–HCl (pH 2.0). The concentrations of rutin and components *X* and *Y* are expressed as in Fig. 2. ●, rutin; △, component *X*; □, component *Y*.

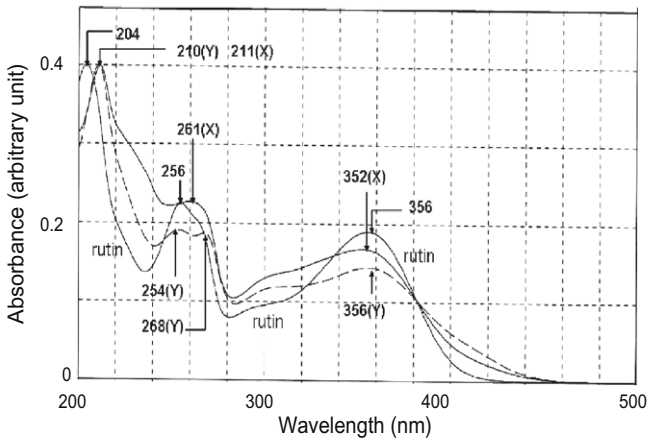

Fig. 5. Absorption spectra of rutin and components *X* and *Y*. The spectra were recorded in the mobile phase for HPLC.

around 300 and 420 nm and decreased at about 255 and 350 nm. An isosbestic point was observed at about 313 nm. The absorption increase around 420 nm was suppressed by SCN^-, but SCN^- enhanced the absorption increase around 300 nm. An isosbestic point was observed at 327 nm in the presence of SCN^-. The SCN^--dependent suppression suggests that SCN^- inhibited the formation of the quinone form of rutin. The formation of the quinone in the absence

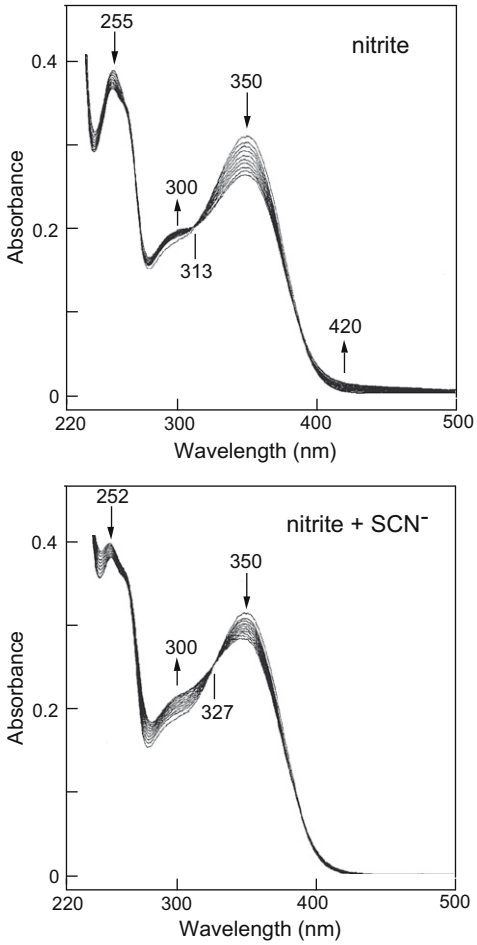

Fig. 6. Effects of thiocyanate on nitrite-induced changes in absorption spectra of rutin. Upper panel: without 1 mM NaSCN. Lower panel: with 1 mM NaSCN. Scanning was repeated every 1.1 min from 500 to 220 nm.

of SCN^- was supported by the result that the concentration of rutin was increased by the addition of ascorbic acid to the reaction mixture, in which a part of rutin had been transformed to its quinone form (not shown). The oxidation of rutin to its *o*-quinone form has been reported in a horseradish peroxidase/rutin/H_2O_2 system under acidic conditions (Takahama, 1986).

3.2. Identification of component Y

Component *Y* isolated had the following characteristics. UV spectrum [methanol and 25 mM KH_2PO_4 (pH 4.5) (1:1, v/v)], λ max, 210, 254, 268, 356 nm: ESI-MS (negative mode), *m*/*z* 667.1 ($[M-H]^-$); (positive mode), 691.1 ($[M+Na]^+$), 669.1 ($[M+H]^+$), 523, 361: APCI-MS (negative mode), 667.1 ($[M-H]^-$); (positive mode), 669.1 ($[M+H]^+$). ^1H NMR (DMSO-d_6) δ 0.98 (d, J = 6.4 Hz, 3H, H-6‴), 3.06 (m, 1H, H-4″), 3.08 (m, 1H, H-4‴), 3.20 (m, 1H, H-3″), 3.21 (m, 1H, H-2″), 3.24 (m, 1H, H-5‴), 3.25 (m, 1H, H-3‴), 3.26 (m, 1H, H-6″a), 3.29 (m, 1H, H-5″), 3.41 (m, 1H, H-2‴), 3.70 (m, 1H, H-6″b), 4.37 (s, 1H, H-1‴), 5.33 (d, J = 7.3 Hz, 1H, H-1″), 6.25 (d, J = 2.0 Hz, 1H, H-6), 6.45 (d, J = 2.0 Hz, 1H, H-8), 7.03 (d, J = 9.3 Hz, 1H, H-5′), 8.22 (d, J = 8.8 Hz, 1H, H-6′), 12.39 (s, 1H, 5-OH). ^{13}C NMR (DMSO-d_6) δ 17.59 (C-6‴), 66.67 (C-5″), 68.12 (C-5‴), 69.74 (C-4″), 70.25 (C-2‴), 70.50 (C-3‴), 71.64 (C-4‴), 73.94 (C-2″), 75.71 (C-5″), 76.17 (C-3″), 93.36 (C-8), 99.05 (C-6), 100.57 (C-1‴), 101.40 (C-1″), 103.88 (C-4a), 114.52 (C-5′), 115.24 (C-1′), 122.94 (C-2′), 128.26 (C-6′), 134.02 (C-3), 136.27 (C-3′), 144.72 (C-4′), 153.18 (C-2), 155.68 (C-8a), 161.20 (C-5), 164.49 (C-7), 168.57 (*C*=O), 177.09 (C-4). The data of elemental analysis were as following: Anal. Calcd for $C_{28}H_{28}O_{17}S$ 2.5 H_2O: C, 47.13; H, 4.66. Found: C, 46.08; H, 4.55. From the above data, the structure of component *Y* obtained in this study was determined to be 5,7-dihydroxy-2-(7-hydroxy-2-oxobenzo[*d*][1,3]oxathiol-4-yl)-4*H*-chromen-4-one-3-*O*-β-rutinoside as shown in Fig. 1 with molecular mass ($C_{28}H_{28}O_{17}S$) of 668.15 and the purity was calculated to be about 97%.

3.3. Significance of the formation of oxathiolone compound

The formation of an oxathiolone compound has been reported in the mixture of chlorogenic acid, nitrite and SCN^- under acidic conditions (Takahama et al., 2007). Taking the mechanism of the formation of the oxathiolone compound from chlorogenic acid into consideration, we deduced that SCN^- might be, at first, reacted with the quinone form of rutin producing 2′-thiocyanaterutin and that the conjugate was hydrolysed producing the oxathiolone fused rutin and NH_3 (Fig. 7). The formation of an oxathiolone compound from *p*-benzoquinone has been previously reported (Konieczny et al., 2007). If rutin was transformed to the quinone form, the reduction of nitrous acid to NO should also occur. Then, we estimated the generation of NO using an O_2 electrode as it has been reported that NO produced can be detected by the decrease in concentration of O_2 due to the auto-oxidation (Lewis & Deen, 1994; Takahama et al., 2003; Venkataraman, Martin, Schafer, and Buettner, 2000) and that an acidic mixture of *o*-diphenol and nitrite produces NO consuming O_2 (Takahama, Tanaka, & Hirota, 2008). The result shows that rutin enhanced nitrite-induced O_2 uptake in 50 mM KCl–HCl (pH 2.0) (data not shown), indicating rutin-dependent reduction of nitrous acid to NO.

The quinone form of rutin may be toxic as quinones can react with components with SH groups (Moridani, Scobie, Jamshidzadeh, Salehi, & O'brien, 2001) and as the semiquinone formed by the coupling of an *o*-diphenol and its quinone form can produce OH radicals by reacting with H_2O_2 (Halliwell & Gutteridge, 1999). In the stomach, the toxic quinone can be effectively reduced to rutin by ascorbic acid (Fig. 7), which is contained in gastric juice (McColl, 2005). When ascorbic acid has been extinguished, the quinone form of rutin can react with SCN^- producing the thiocyanate conjugate, which transforms to the oxathiolone derivative of rutin. In this way, the stomach is protected from the toxic effects of *o*-quinones by reducing them by ascorbic acid and by forming oxathiolone compounds even when toxic quinones were formed (Fig. 7).

When quercetin was used instead of rutin, nitrite might induce the formation of a stable oxidation product of quercetin, 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(*2H*)-benzofuranone, under acidic conditions (Hirota, Takahama, Ly, & Yamauchi, 2005; Takahama et al., 2002). The formation seemed not to be affected by SCN^-. The result suggests that SCN^- could not react with the quinone form of quercetin that might be formed as an intermediate during the formation of the above stable compound (Braune, Gütschow, Engst, & Blaut, 2001; Kubo, Nihei, and Shimizu, 2004). The reason for the failure of reaction of the quinone form of quercetin with SCN^- may be due to the much faster reaction of the quinone form with water than SCN^-.

3.4. Perspectives

It is suggested in the present study that oxathiolone compounds can be formed in the stomach when foods or beverages that contain *o*-diphenols such as rutin and chlorogenic acid are ingested. During the formation of oxathiolone compounds, NO may be generated and toxic *o*-quinones may be scavenged by SCN^-. Recently, compounds with an oxathiolone moiety in the benzene ring have been shown to have antifungal, bacteriostatic and cytostatic activities (Konieczny et al., 2007) and to be able to inhibit κB kinase β (Kim et al., 2008). These reports suggest that the oxathiolone compounds formed from rutin and chlorogenic acid may also have some pharmacological functions by themselves and/or after removing their sugar moiety by hydrolysis by intestinal bacteria. Further studies are required to elucidate the functions of components with an oxathiolone moiety, which are formed from phenolic components in foods and beverages in the stomach.

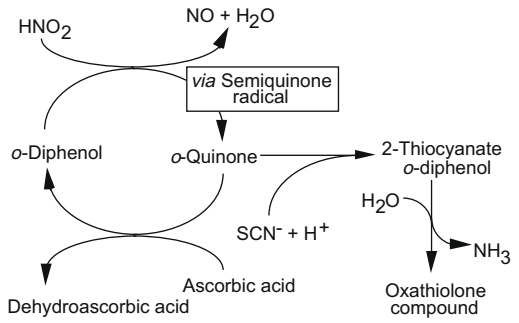

Fig. 7. Scheme of the formation of *o*-quinone and oxathiolone compound in the stomach.

References

Braune, A., Gütschow, M., Engst, W., & Blaut, M. (2001). Degradation of quercetin and luteolin by *Eubacterium ramulus*. *Applied and Environmental Microbiology, 67*, 5558–5567.

Danila, A.-M., Kotani, A., Hakamata, H., & Kusu, F. (2007). Determination of rutin, catechin, epicatechin, and epicatechin gallate in buckwheat *Fagopyrum esculentum* Moench by micro-high-performance liquid chromatography with electrochemical detection. *Journal of Agricultural and Food Chemistry, 55*, 1139–1143.

Gago, B., Lundberg, J. O., Barbosa, R. M., & Laranjinha, J. (2007). Red wine-dependent reduction of nitrite to nitric oxide in the stomach. *Free Radical Biology and Medicine, 43*, 1233–1242.

Halliwell, B., & Gutteridge, J. M. C. (1999). *Free radical in biology and medicine*. Oxford: Oxford University Press.

Hirota, S., Takahama, U., Ly, T. N., & Yamauchi, R. (2005). Quercetin-dependent inhibition of nitration induced by peroxidase/H_2O_2/nitrite systems in human

saliva and characterization of an oxidation product of quercetin formed during the inhibition. *Journal of Agricultural and Food Chemistry, 53*, 3265–3272.

Ihme, N., Kiesewetter, H., Jung, H., Hoffmann, K. H., Birk, A., Müller, A., et al. (1996). Leg oedema protection from a buckwheat herb tea in patients with chronic venous insufficiency: A single-centre, randomised, double-blind, placebo-controlled clinical trial. *European Journal of Clinical Pharmacology, 50*, 443–447.

Kim, B. H., Roh, E., Lee, H. Y., Lee, I. J., Ahn, B., Jung, S. H., et al. (2008). Benzoxathiole derivative blocks lipopolysaccharide-induced nuclear factor-kappaB activation and nuclear factor-kappaB-regulated gene transcription through inactivating inhibitory kappaB kinase beta. *Molecular Pharmacology, 73*, 1309–1318.

Konieczny, M. T., Konieczny, W., Sabisz, M., Skladanowski, A., Wakiec, R., Augustynowicz-Kopec, E., et al. (2007). Synthesis of isomeric, oxathiolone fused chalcones, and comparison of their activity toward various microorganisms and human cancer cells line. *Chemical and Pharmaceutical Bulletin, 55*, 817–820.

Kreft, I., Fabjan, N., & Yasumoto, K. (2006). Rutin content in buckwheat (*Fagopyrum esculentum* Moench) food materials and product. *Food Chemistry, 98*, 508–512.

Kreft, S., Knapp, M., & Kreft, I. (1999). Extraction of rutin from buckwheat (*Fagopyrum esculentum* Moench) seeds and determination by capillary electrophoresis. *Journal of Agricultural and Food Chemistry, 47*, 4649–4652.

Kubo, I., Nihei, K., & Shimizu, K. (2004). Oxidation products of quercetin catalyzed by mushroom tyrosinase. *Bioorganic and Medical Chemistry, 12*, 5343–5347.

Lewis, R. S., & Deen, W. M. (1994). Kinetics of the reaction of nitric oxide with oxygen in aqueous solution. *Chemical Research in Toxicology, 7*, 568–574.

McColl, K. E. L. (2005). When saliva meets acid: Chemical warfare at the oesophagogastric junction. *Gut, 54*, 1–3.

Moridani, M. Y., Scobie, H., Jamshidzadeh, A., Salehi, P., & O'brien, P. J. (2001). Caffeic acid, chlorogenic acid, and dihydrocaffeic acid metabolism: Glutathione conjugate formation. *Drug Metabolism and Disposition: The Biological Fate of Chemicals, 29*, 1432–1439.

Ölschläger, C., Regos, I., Zeller, F. J., & Treutter, D. (2008). Identification of galloylated propelargonidins and procyanidins in buckwheat grain and quantification of rutin and flavanols from homostylous hybrids originating from *F. Esculentum x F. homotropicum. Phytochemistry, 69*, 1389–1397.

Pannala, A. S., Mani, A. R., Spencer, J. P., Skinner, V., Bruckdorfer, K. R., Moore, K. P., et al. (2003). The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. *Free Radical Biology and Medicine, 34*, 576–584.

Peri, L., Pietraforte, D., Scorza, G., Napolitano, A., Fogliano, V., & Minetti, M. (2005). Apples increase nitric oxide production by human saliva at the acidic pH of the stomach: A new biological function for polyphenols with a catechol group? *Free Radical Biology and Medicine, 3*, 668–681.

Takahama, U. (1986). Spectrophotometric study on the oxidation of rutin by horseradish peroxidase and characteristics of the oxidation products. *Biochimica et Biophysica Acta, 882*, 445–451.

Takahama, U., & Hirota, S. (2008). Reduction of nitrous acid to nitric oxide by coffee melanoidins and enhancement of the reduction by thiocyanate: Possibility of its occurrence in the stomach. *Journal of Agricultural and Food Chemistry, 56*, 4736–4744.

Takahama, U., Hirota, S., Yamamoto, A., & Oniki, T. (2003). Oxygen uptake during the mixing of saliva with ascorbic acid under acidic conditions: Possibility of its occurrence in the stomach. *FEBS Letters, 550*, 64–68.

Takahama, U., Oniki, T., & Hirota, S. (2002). Oxidation of quercetin by salivary components. Quercetin-dependent reduction of salivary nitrite under acidic conditions producing nitric oxide. *Journal of Agricultural and Food Chemistry, 50*, 4317–4322.

Takahama, U., Tanaka, M., & Hirota, S. (2008). Interaction between ascorbic acid and chlorogenic acid during the formation of nitric oxide in acidified saliva. *Journal of Agricultural and Food Chemistry, 56*, 10406–10413.

Takahama, U., Tanaka, M., Oniki, T., Hirota, S., & Yamauchi, R. (2007). Formation of the thiocyanate conjugate of chlorogenic acid in coffee under acidic conditions in the presence of thiocyanate and nitrite: Possible occurrence in the stomach. *Journal of Agricultural and Food Chemistry, 55*, 4169–4176.

Venkataraman, S., Martin, S. M., Schafer, F. Q., & Buettner, G. R. (2000). Detailed methods for the quantification of nitric oxide in aqueous solutions using either an oxygen monitor or EPR. *Free Radical Biology and Medicine, 29*, 580–585.

Watanabe, M., Ohshita, Y., & Tsushida, T. (1997). Antioxidant compounds from buckwheat (*Fagopyrum esculentem* Moench) hulls. *Journal of Agricultural and Food Chemistry, 45*, 1039–1044.