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Reactions of polyphenols in masticated apple fruit with nitrite under stomach simulating conditions: Formation of nitroso compounds and thiocyanate conjugates

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Chemical compounds studied in this article:
(+)-Catechin (PubChem CID: 9064)
(−)-Epicatechin (PubChem CID: 72276)
Procyanidin B1 (CID: 11250133)
Procyanidin B2 (PubChem CID: 122738)
Procyanidin B5 (CID: 124017)
Chlorogenic acid (CID: 1794427)

ABSTRACT

By the ingestion of fresh apple fruit, it is masticated squeezing apple juice into the oral cavity and the juice is mixed with saliva. The mixture of saliva and apple juice is swallowed into the stomach where the pH is around 2. This paper deals with the reactions of polyphenols in the juice obtained by mastication of apple fruit with salivary nitrite under acidic conditions. The concentrations of catechins, procyanidins, and chlorogenic acid in the apple juice were approximately 55, 55, and 170 μM, respectively, and the polyphenols were oxidized by salivary nitrite under conditions of the stomach. Rates of the oxidation increased in order chlorogenic acid < catechins < procyanidins. The oxidation of catechins and procyanidins resulted in the formation of the nitroso compounds, and the oxidation of chlorogenic acid resulted in the formation of the thiocyanate conjugate. The production of dinitroso compounds is proposed to be due to the addition of nitric oxide (•NO) to radicals of catechins and procyanidins. As the mechanism of thiocyanate conjugate formation, reaction of *o*-quinone of chlorogenic acid with a salivary component thiocyanate is proposed, and the formation of the thiocyanate conjugate is discussed from the point of detoxification of chlorogenic acid quinone.

1. Introduction

Apple fruit contains polyphenols such as chlorogenic acid, (−)-epicatechin, and procyanidin B2 (Fig. 1), and their concentrations have been reported to be approximately 0.4, 0.2 and 0.1 μmol/g of fresh weight, respectively (Hirota & Takahama, 2014). In addition to these polyphenols, (+)-catechin, procyanidin B1, and procyanidin B5 are also present in apple fruit (Foo & Lu, 1999; Shoji et al., 2003; Xiao et al., 2008) (Fig. 1). In the acidic mixture of mixed whole saliva and the methanol extract of apple, which is simulated the gastric lumen, the consumption of (−)-epicatechin and procyanidin B2 is observed (Hirota & Takahama, 2014; Peri et al., 2005). The consumption results in the transformation of (−)-epicatechin and procyanidin B2 into 6,8-dinitrosoepicatechin and dinitrosoprocyanidin B2, respectively (Lee et al., 2006). The formation of 6,8-dinitrosoepigallocatechin gallate from epigallocatechin gallate/nitrous acid systems has been reported (Panzella, Manini, Napolitano, & d'Ischia, 2005). As the mechanism of the nitrosation, the reaction of catechin semiquinone radicals with nitric oxide (•NO) is proposed (Hirota & Takahama, 2014; Takahama, Yamauchi, & Hirota, 2014; Veljovic-Jovanovic, Morina, Yamauchi, Hirota, & Takahama, 2014). Both •NO and the semiquinone radicals can be produced in a catechin/nitrous acid system (Peri et al., 2005).

In contrast to catechins, the consumption of chlorogenic acid, a major polyphenol in apple fruit, is slow when methanol extracts of apple fruit were added to acidified mixed whole saliva (Hirota & Takahama, 2014). The consumption does not accompany the nitrosation of chlorogenic acid (Hirota & Takahama, 2014), although both •NO and semiquinone radical of chlorogenic acid are produced in chlorogenic acid/nitrous acid systems (Peri et al., 2005). The nitration of chlorogenic acid in chlorogenic acid/nitrous acid systems has been reported (Cotelle & Vezin, 2001). In addition to the nitration, chlorogenic acid is oxidized to its quinone by nitrous acid, and the quinone is transformed into a thiocyanate conjugate that is hydrolyzed to the oxathiolone derivative and NH_3 (Takahama, Tanaka, Oniki, Hirota, & Yamauchi, 2007). The formation of thiocyanate

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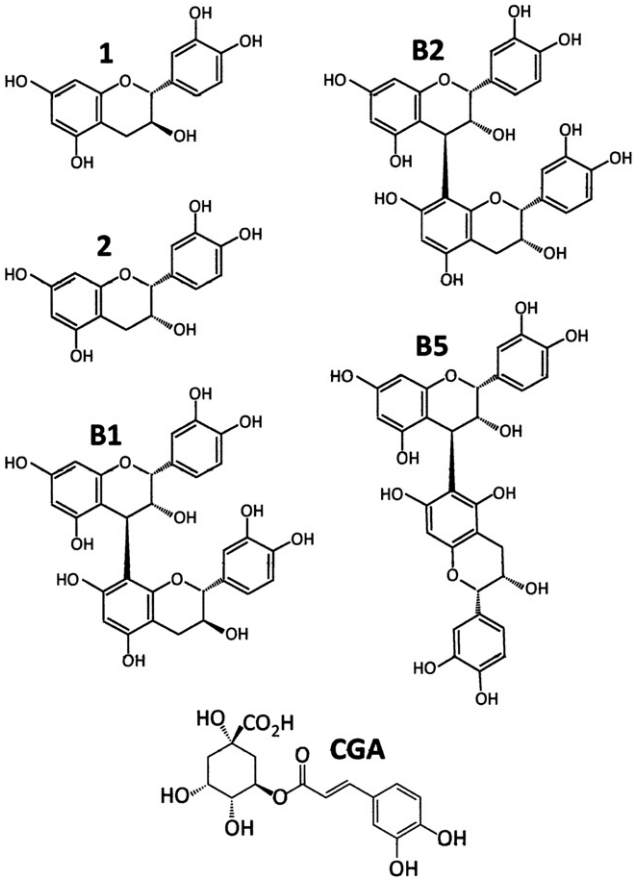

Fig. 1. Compounds concerned in this study. **1**, (+)-catechin; **2**, (−)-epicatechin; **B1**, procyanidin B1; **B2**, procyanidin B2; **B5**, procyanidin B5; **CGA**, chlorogenic acid.

conjugate is discussed in relation to the detoxification of chlorogenic acid quinone in the stomach (Takahama et al., 2007).

This paper deals with the reactions of nitrite with apple polyphenols in the juice, which is produced in the oral cavity by the mastication of apple fruit, under the conditions simulating the gastric lumen. Taking the result obtained in this study into account, we discuss (i) the possible reactions of apple polyphenols with salivary nitrite, (ii) the possible function of salivary thiocyanate, and (iii) the importance of elucidation of the chemical reactions in the stomach.

2. Materials and methods

2.1. Reagents and material

Procyanidin B1 was obtained from Tokiwa Phytochemical Co., Ltd. (Sakura, Japan). Procyanidin B2 and (−)-epicatechin were obtained from Extrasynthese (Lyon, France) and Sigma-Aldrich Japan (Tokyo), respectively. Chlorogenic acid, (+)-catechin, and Griess-Romijin reagent for nitrite were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Apple (cultivar sanfuji) was obtained from a local market in Kitakyushu. Thiocyanate conjugate and oxathiolone of chlorogenic acid were prepared as reported previously (Takahama et al., 2007).

2.2. Methanol extraction of apple fruit polyphenols

One gram of apple fruit was homogenized in 3 mL of methanol using a pestle and a mortar. After centrifugation of the homogenate at 3000 *g* for 2 min, the sediment was suspended in 6 mL of methanol. The methanol suspension was centrifuged again under the above conditions. The supernatants obtained by the first and the second centrifugations were combined. After evaporating the solvents in vacuo, the residue was dissolved in 1 mL of 20% (v/v) methanol in 0.2% (v/v) formic acid. The solution was filtered with a membrane filter (pore size, 0.45 μm) (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to be analyzed by HPLC.

2.3. Preparation of apple juice by mastication

After their informed consent had been obtained, two non-smoking healthy volunteers agreed to help our experiments. Peeled apple fruit (10 g) was masticated for 15 s around 9 a.m., being careful not to swallow the juice squeezed during the mastication. The masticated apple fruit was spitted out on four layers of gauze to prepare the juice. Volume of the juice was 6–8 mL and the pH was 4.0–4.5. Saliva obtained by chewing parafilm for 15 s was 0.4–0.9 mL ($n = 4$). The volumes suggest that saliva content in the juice was approximately 10% (v/v).

2.4. Determination of concentration of nitrite and thiocyanate in apple juice

A portion (about 1 mL) of the apple juice obtained by mastication was passed through the above membrane filter, and the nitrite concentrations were determined using Griess-Romijin reagent as reported previously (Takahama, Hirota, Yamamoto, & Oniki, 2003). To quantify thiocyanate, 1 mL of the apple juice was extracted with 5 mL of ethyl acetate, and the aqueous phase was passed through the above membrane filter. An aliquot of the filtrate (20 μL) was analyzed by HPLC (Takahama, Tanaka, Oniki, & Hirota, 2007) (see below).

2.5. Reaction of apple juice polyphenols with nitrous acid

After the ingestion of apple, the pH of masticated apple decreases to about 2 in the stomach and salivary nitrite is continuously provided to the gastric lumen. It has been reported that the half time of gastric emptying is around 2 h after the ingestion of foods (Camilleri et al., 1989; Degen & Phillips, 1996). Then, the pH of apple juice obtained as described above was decreased to 2.0 by adding 20–25 μL of 2 M HCl. Immediately after the decrease in pH, (i) the juice was divided into test tubes (1 mL each), (ii) various concentrations of sodium nitrite were added to the juice, and then (iii) the juice was incubated for defined periods. After the incubation, the incubated juice was extracted with 5 mL of ethyl acetate. Ethyl acetate of the extract was evaporated in vacuo, and the residue was dissolved in 1 mL of 20% (v/v) methanol in 0.2% (v/v) formic acid. An aliquot (0.1 mL) of the solution was applied to an analytical HPLC column.

2.6. Formation of thiocyanate conjugate of chlorogenic acid

Acidified apple juice (1 mL) was incubated with various concentrations of nitrite with and without 1 mM thiocyanate. After the incubation for 10 min, the juice was extracted with ethyl acetate as described above. The residues obtained after the evaporation of ethyl acetate in vacuo were dissolved in 20% (v/v) methanol in 0.2% (v/v) formic acid to quantify the thiocyanate conjugate produced.

2.7. Analytical HPLC

Analytical HPLC was performed using a Shim-pack CLC-ODS column (15 cm × 6 mm i.d.) combined with a pump (LC-6A) and a spectrophotometric detector with a photodiode array (SPD-M10Avp) (Shimadzu, Kyoto, Japan). The mobile phases to separate and quantify polyphenols were mixtures of methanol and 0.2% (v/v) formic acid (1:4, 1:3, or 2:5, v/v), and their flow rate was 1 mL/min. Polyphenols and the reaction products were quantified from the areas under the peaks at 320 nm for chlorogenic acid, nitroso compounds of catechins, and procyanidins, and at 210 or 280 nm for catechins and procyanidins (Veljovic-Jovanovic et al., 2014).