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Conversion to purpurogallin, a key step in the mechanism of the potent xanthine oxidase inhibitory activity of pyrogallol

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ABSTRACT

In this study, the mechanism of the xanthine oxidase (XO) inhibitory activity of pyrogallol, the main inhibitor found in roasted coffee, was investigated. Pyrogallol was unstable and readily converted to purpurogallin in a pH 7.4 solution, a physiological model of human body fluids. The XO inhibitory activity of the produced purpurogallin was higher than that of pyrogallol, as evidenced by comparing their IC_{50} values (0.2 μmol L^{-1} for purpurogallin, 1.6 μmol L^{-1} for pyrogallol). The XO activity of pyrogallol was enhanced by pre-incubation in pH 7.4 solution. Although the initial XO inhibitory activity of 4-methylpyrogallol was weak (IC_{50} 33.3 μmol L^{-1}), its XO inhibitory activity was also enhanced by pre-incubation in the pH 7.4 solution. In contrast, 5-methylpyrogallol, which could not be transformed into corresponding purpurogallin derivatives, did not show XO inhibitory activity before or after incubation in pH 7.4 solution. Molecular docking simulations clarified that purpurogallins have stronger affinities for XO than corresponding pyrogallols. These results revealed that the potent XO inhibitory activity seemingly observed in pyrogallol is actually derived from its chemical conversion, under alkaline conditions, into purpurogallin.

1. Introduction

Xanthine oxidase (XO), the final enzyme in purine catabolism of humans, catalyses the oxidation reactions of hypoxanthine and xanthine. The reactions produce uric acid and reactive oxygen species such as superoxide and hydrogen peroxide. It is well known that the onset of gout is due to an over-accumulation of plasma uric acid: this over-accumulation is believed to be related to eating habits [1]. Therefore, in spite of being a classical disease, gout is now also recognized as a current lifestyle-related disease, with recent increases in the number of patients experiencing it [2]. Cardiovascular disease is another lifestyle-related disease, and it is well known to be related to oxidative stress caused by superoxide, hydrogen peroxide, nitric oxide, and their metabolites (hydroxyl radical, peroxynitrite, etc.) [3]. Thus, inhibition of the over-working of XO is important in preventing such lifestyle-related diseases [4]. For gout treatment, several synthetic XO inhibitors were developed in the 1950s and1960s; however, they are toxic to human organs [5]. Therefore, several recent investigations have sought safer and more effective XO inhibitors from natural sources, including foods and traditional herbs [6]. Coffee is the most popular beverage in the world; it is comes from the beans of tropical *Caffea arabica* trees (and related species), and is made by roasting, grinding, and brewing the beans. In epidemiological studies published in 2007, Choi et al. [7] reported that a coffee drinking habit significantly reduces the incidence of gout. They also showed that coffee reduced uric acid levels in the serum of in coffee drinkers [8,9]. These results indicated that coffee could be a promising source of the XO inhibitors, and prompted us to seek those inhibitory compounds in coffee as candidates for medicines to treat gout. In a previous study, we observed that XO-inhibitory activity only in roasted coffee beans, and not in green or dried beans [10]. We have also isolated XO inhibitors from hot-water extracts of roasted coffee beans, and identified them as pyrogallol and several chlorogenic lactones [11]. Based on quantitative analyses and evaluations of the respective activity efficiencies of XO inhibitors, we hypothesized that pyrogallol was the main contributor of the XO-inhibitory activities in roasted coffee.

Pyrogallol (1,2,3-trihydroxybenzene) is a simple polyphenol that bears the decarboxylated structure of gallic acid, which itself is distributed widely in plants as a component of hydrolysable tannin. Polyphenols have high reactivity for radical trapping and reducing reactions [12], and this reactivity is enhanced under alkaline conditions [13]. Therefore, the high reactivity and resulting structural changes of

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pyrogallol must be studied chemically to clarify its XO-inhibitory mechanism. In this paper, we report on the relation between pyrogallol's XO inhibition and reactivity at pH 7.4 as a model of physiological conditions, and we discuss the results of our simulations of the molecular docking of pyrogallol (and its related substances) with XO.

2. Materials and methods

2.1. Materials

Pyrogallol, 5-methylpyrogallol, and 2,3,4-trihydroxybenzaldehyde were purchased from Tokyo Kasei (Tokyo, Japan). Sodium cyanoborohydride was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Xanthine oxidase from buttermilk (ca. 0.3 units mg^{-1}) was obtained from Oriental Yeast (Tokyo, Japan). All other reagents and solvents were purchased as extra pure grade or HPLC grade from Nacalai Tesque (Kyoto, Japan).

2.2. General procedure

NMR spectra were obtained from a JNM-ECZ400S (JEOL, Tokyo, Japan) using the manufacture provided pulse sequences. MS spectra were measured using a JMS-T100 spectrometer (JEOL) with direct analysis at real time (DART) ionization and time of flight (TOF) measuring mode under the following conditions: orifice 1, 10 V; orifice 2, 5 V; ring lens, 10 V; helium gas pressure, 0.5 MPa, flow rate of 10 L min^{-1}; ion resource temperature, 350 °C; grid voltage positive, 400 V for positive mode. Elemental compositions of analyzed compounds were calculated using MassLynx software (v. 4.1, Waters, MA, USA). Analytical HPLC was performed using a PU-4180 quaternary gradient pump (JASCO, Tokyo, Japan) equipped with an MD-4015 photodiode array detector (JASCO), a CTO-10ASVP column oven (Shimadzu, Kyoto, Japan), and an AS-4150 intelligent sampler (JASCO). Data were analyzed using the ChromNAV software (v.2, JASCO). Preparative HPLC was performed using an LC-6 AD pump (Shimadzu) equipped with an SPD-6A UV detector (Shimadzu).

2.3. Assay for xanthine oxidase inhibition

The XO inhibitory assay was carried out according to a previously reported method, with modification [14]. The reaction medium consisted of 10 μL of 1 mmol L^{-1} xanthine in DMSO, and 160 μL of 12.5 mmol L^{-1} phosphate buffer (pH 7.4). This solution was pre-heated at 37 °C for 5 min. To this solution was added 10 μL of the test sample in DMSO, immediately followed by the addition of 20 μL of 0.027 unit mL^{-1} XO solution (pH 7.4 phosphate buffer). After incubation at 37 °C for 10 min, 25 μL of 3% $HClO_4$ in aqueous solution was added to quench the reaction. An aliquot (20 μL) of the solution was injected into an HPLC column to quantify the uric acid produced. The HPLC analysis was performed under the following conditions. Column, 250 mm × 4.6 mm, i.d., 5 μm, Mightysil RP-18 GP Aqua (Kanto Chemical, Tokyo, Japan); flow rate, 1.0 mL min^{-1}; solvent, [methanol − 0.1% phosphoric acid in water (2.5:97.5, v/v)]; detection wavelength, 290 nm; temperature, 35 °C. Percent inhibition was calculated according to the following equation: inhibition (%) = [(peak area of uric acid in control experiment) − (peak area of uric acid in sample experiment)] × 100/(peak area of uric acid in control experiment).

2.4. Stability test and measurements of pyrogallol's XO-inhibitory activity at various pH conditions

At 37 °C, 10 μL of pyrogallol in H_2O (4 mmol L^{-1}) was added to 190 μL of phosphate buffer solution (12.5 mmol L^{-1}) under various pH conditions. An aliquot (20 μL) was taken from the solution immediately after addition of pyrogallol and again after 10 min had elapsed. The solution was analyzed via HPLC under the following conditions. Column, Cosmosil 5C18-AR-II (4.6 × 250 mm); solvent, [0.1% phosphoric acid in water-CH_3CN (95:5, v/v)]; flow rate, 1.0 mL min^{-1}; detection wavelength: 268 nm. These procedures were repeated using each of several phosphate buffer solutions of different pHs and the XO inhibitory activity of each solution was measured by the same procedure described above.

2.5. Preparation and identification of chemicals

2.5.1. 4-Methylpyrogallol

To an acetic acid solution (10 mL) of 2,3,4-trihydroxybenzaldehyde (0.5 g) was added sodium cyanoborohydride (0.5 g) at 23 °C, and then the solution was heated at 60 °C with stirring for 2 h. After the solvent was removed under reduced pressure at 40 °C, the obtained residue was purified by silica gel column chromatography (NAM-300H, Nagara Science, Gifu, Japan) eluted with CH_2CH_2-CH_3OH = 20:1–10:1 to give 4-methylpyrogalol (0.21 g). ^1H NMR (400 MHz in acetone-d_6) δppm 2.14 (3H, s, 4-CH_3), 6.32 (1H, d, *J* = 8.0 Hz, H5), 6.45 (1H, d, *J* = 8.0 Hz, H6), 7.13 (1H, brs, OH), 7.26 (1H, brs, OH), 7.85 (1H, brs, OH); DART-MS *m/z* 141 $[M+H]^+$.

2.5.2. Purpurogallin

Preparation was carried out according to a reported method [15]. To a suspension of pyrogallol (2 g) in small amount of H_2O was dropwise added $NaIO_3$ (1.6 g) in H_2O (20 mL) at 0 °C. After addition, the dark red precipitate formed was filtered. The red powder was recrystallized with acetic acid to give purpurogallin (1.0 g). UV–vis (in CH_3CN-H_2O), λmax (nm) 280, 300, 368, and 428; DART-MS *m/z* 221 $[M+H]^+$; ^1H NMR (400 MHz in DMSO-d_6) δppm 6.72 (1H, dd, *J* = 15.2 and 12.4 Hz, H8), 6.86 (1H, s, H1), 7.07 (1H, d, *J* = 12.4 and 1.2 Hz, H7), 7.29 (1H, brd, H9).

2.5.3. 1,7-Dimethylpurpurogalin

4-Methylpyrogallol (100 mg) was dissolved in a phosphate buffer (pH 7.4, 71 mL) and then stirred at 40 °C for 1 day. Red precipitated formed was filtered to give 27 mg of 1,7-dimethylpurpurogallin. UV–vis (in CH_3CN-H_2O), λmax(nm) 284, 308, 384, and 440; DART-MS *m/z* 249 $[M+H]^+$; ^1H NMR (400 MHz in CD_3OD) δppm 2.37 (3H, s, 7-CH_3), 2.42 (3H, s, 1-CH_3), 6.78 (1H, d, *J* = 12.4 Hz, H8), 7.53 (1H, d, *J* = 12.4 Hz, H9). NOEs were observed 1.7% between the peaks at 2.37 and 6.78 ppm, 0.5% between the peaks at 2.37 and 7.53 ppm, and 4.2% between the peaks at 2.42 and 7.53 ppm.

2.6. Time-course analysis of the conversion of pyrogalolls to purpurogallins and XO-inhibitory activity of the reaction solutions

To 950 μL of phosphate buffer (12.5 mmol L^{-1}, pH 7.4), 50 μL of DMSO pyrogalloll or related compounds-solution (consisting of 400 μmol L^{-1} pyrogaloll, 4000 μmol L^{-1} 4-methylpyrogallol, or 4000 μmol L^{-1} 5-methylpyrogallol) was added, and the solution was incubated at 37 °C for 4 h. At different time intervals (0, 0.5, 1, 2, and 4 h), a 160 μL aliquot was taken from the solution and then acidified by adding 3% $HClO_4$ aq. (16 μL). Twenty μL of the solution was analyzed by HPLC under following conditions. Column, 250 mm × 4.6 mm, i.d., 5 μm, Cosmosil 5C18-AR-II (Nacalai); flow rate, 1.0 mL min^{-1}; solvent A, [0.1% phosphoric acid in water]; solvent B, acetonitrile; gradient conditions, linear gradient of solvent B (5–45%, 0–40 min), then 100% (40–50 min), and then retention of 100% solvent B (50–55 min); detection wavelength, 268, 280, 300, and 428 nm. Produced purpurogallins were identified by comparing retention times and UV–vis spectra of specific peaks with the authentic samples synthesized as stated above.

Pyrogallols and purpurogallins in the reaction solutions were quantified via the external standard method, using the following equations. For pyrogallol, y = 26,747x – 1813 (where y = peak area at 268 nm, and x = amount of pyrogallol, ranging 0.25–5 nmol). For 4-