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Characterisation of the antioxidant activity of flavonoids

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ABSTRACT

Inhibition of the superoxide anion (O_2^-) generation catalysed by xanthine oxidase using certain flavonoids was examined to determine their antioxidant effects. All of the flavonoids and their glycosides, except for kaempferol-3-glucoside, considerably and markedly inhibited O_2^- generation. Flavonoids also inhibited uric acid formation, whereas their glycosides did not. The flavonoids and their glycosides with scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl radical had more than one conjugated en-diol group and exhibited more potent inhibition of the O_2^- generation than the uric acid formation catalysed by xanthine oxidase. The results show that the inhibition of O_2^- generation with flavonoids without any conjugated en-diol group was due to competitive inhibition of uric acid formation, while the inhibition with flavonoids having more than one conjugated en-diol group was due to the reduced form of the enzyme.

1. Introduction

Xanthine oxidase is generated from xanthine dehydrogenase by oxidative stress, as in cases of ischaemia–reperfusion. Xanthine oxidase (EC 1.1.3.22) catalyses the oxidative reaction of xanthine (or hypoxanthine) with oxygen to uric acid and two active oxygen species, hydrogen peroxide and superoxide anion (O_2^-), as normal products (Bray, 1975). The one-electron reduction products of O_2, the superoxide anion (O_2^-), and subsequently hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\cdot$) derived from H_2O_2 and O_2^-, actively participate in the initiation of lipid peroxidation. As the O_2^- generated by xanthine oxidase lead to peroxidative damage in cells (Frong, McCay, Poyer, Keele, & Misra, 1973), antioxidants containing radical scavengers and enzyme inhibitors are useful in the prevention of postischemic tissue injury (McCord, 1985). Therefore, to identify antioxidants in foods and natural products, antioxidant activity was examined using xanthine oxidase (Aucamp, Gaspar, Hara, & Apostolides, 1997; Toda, Kumura, & Ohnishi, 1991). Gallic acid, caffeic acid, anacardic acid, flavonoids and so on were reported to be antioxidants. We examined antioxidant activity of anacardic acid, pentadecatrienyl salicylic acid, using xanthine oxidase, and indicated that alk(en)yl chain in anacardic acid was associated with hydrophobic binding to xanthine oxidase (Masuoka & Kubo, 2004). To further study the effect of an alkyl side chain in antioxidants, we examined the activity of gallic acid and alkyl gallates, and found that the alkyl gallates with more C_6 chains functioned as inhibitors of xanthine oxidase, while gallic acid and alkyl gallates functioned as reducing agents of the enzyme to catalyse hydrogen peroxide formation (Masuoka, Nihei, & Kubo, 2006).

In this paper, we planned to examine antioxidant activity using xanthine oxidase with flavonoids, since flavonoids intake prevented the risk for the development of cardiovascular diseases and these effects were mainly attributed to the antioxidant and anti-inflammatory properties (Mladenka, Zaltloukalova, Filipsky, & Hrdina, 2010; Rathee et al., 2009). Certain flavonoids were known as strong inhibitors for xanthine oxidase (Lin, Chen, Chen, Liang, & Lin, 2002), but the effects of flavonoids on O_2^- generation in relation to this inhibition has not been investigated. At first, we examined inhibitory activity for O_2^- generation catalysed by xanthine oxidase with flavonoids. The structures of some of the flavonoids used in the experiments are shown in Fig. 1.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), flavones, flavonols and their glycosides were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Dimethyl sulfoxide (DMSO), EDTA, bovine serum albumin (BSA), nitroblue tetrazolium chloride (NBT), phenazine methosulfate (PMS), the reduced form of nicotinamide adenine dinucleotide (NADH) and xanthine oxidase were purchased from

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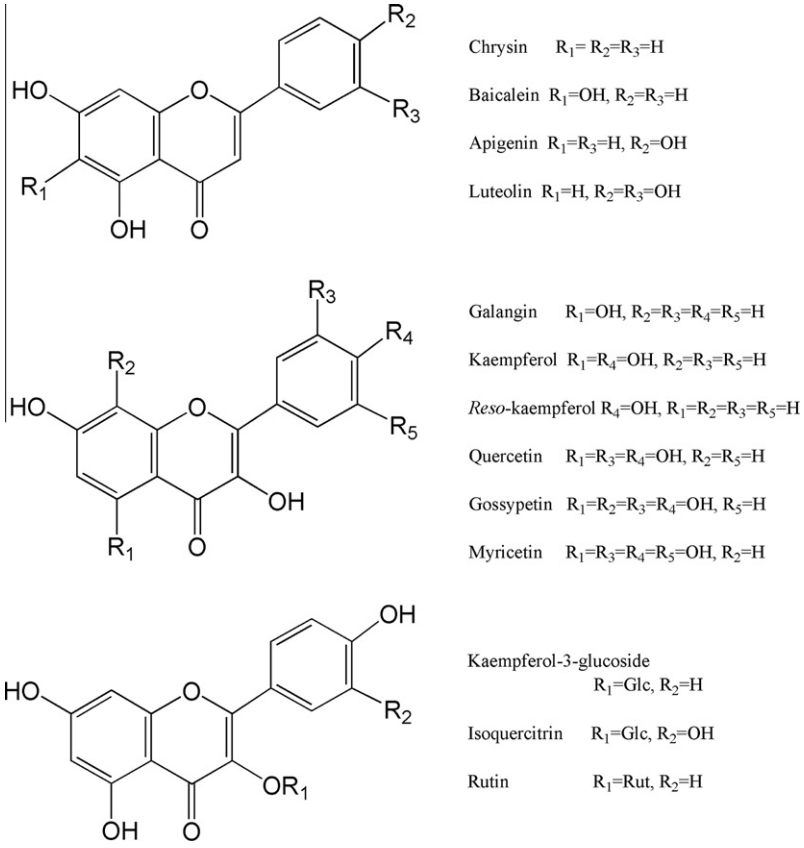

Fig. 1. Structures of certain flavones, flavonols and flavonol glycosides.

the Sigma Chemical Co. (St. Louis, MO). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Assay of O_2^- generated by xanthine oxidase

Xanthine oxidase (EC 1.1.3.22, Grade IV) was purchased from the Sigma Chemical Co. The enzyme reaction was performed thoroughly at pH 10 to detect labile O_2^-. The reaction mixture consisted of 2.70 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 ml 10 mM xanthine, 0.03 ml 0.5% BSA, 0.03 ml 2.5 mM NBT and 0.06 ml sample solution (dissolved in DMSO). To the mixture at 25 °C, 0.12 ml of xanthine oxidase (0.04 U) was added to start the reaction (Toda et al., 1991), and the absorbance at 560 nm was recorded for 60 s by the formation of blue formazan (Masuoka & Kuba, 2004). A control experiment was carried out by replacing the sample solution with the same amount of DMSO.

2.3. Assay of uric acid generated by xanthine oxidase

The reaction mixture consisted of 2.76 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 ml 10 mM xanthine and 0.06 ml sample solution (dissolved in DMSO). The mixture was incubated at 25 °C, before the reaction was started by the addition of 0.12 ml of xanthine oxidase (0.04 U) and the absorbance at 293 nm was recorded for 60 s.

2.4. Radical scavenging activity against the O_2^- generated by the PMS–NADH system

Superoxide anions were generated nonenzymatically with a PMS–NADH system. The reaction mixture (final volume was 3.0 ml) containing 25 μM NBT, 150 μg of BSA, 78 μM NADH and 0.06 ml of sample solution in 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0) was prepared and incubated at 25 °C for 3 min. Afterwards, 0.03 ml of 155 μM PMS was added to start the reaction and the absorbance at 560 nm was recorded for 60 s (Nishikimi, Rao, & Yagi, 1972). As the control, 0.06 ml of DMSO was used. The reaction rate was calculated from the proportional increase of absorbance, and the scavenging activity of the sample was expressed as the IC_{50} value.

2.5. Radical scavenging activity against the DPPH radical

Firstly, 1 ml 100 mM acetate buffer (pH 5.5), 1.87 ml ethanol and 0.1 ml an ethanolic solution of 3 mM DPPH were put into a test tube and incubated at 25 °C. Afterwards, 0.03 ml of the sample solution (dissolved in DMSO) were added to the mixture and absorbance at 517 nm (DPPH, $\varepsilon = 8.32 \times 10^3\ M^{-1}\ cm^{-1}$) was recorded at 0 and 20 min (Blois, 1958). As a control, 0.03 ml of DMSO was added to the mixture. From the decrease (ΔA) of absorbance obtained, scavenging activity was calculated as the number (N) of DPPH molecules scavenged per test molecule as follows (Masuoka & Kuba, 2004):

$$N = (\Delta A_{\text{sample}} - \Delta A_{\text{control}})/(\varepsilon \times C_{\text{sample}}) \quad (1)$$

2.6. Assay and data analysis

Each assay was performed in triplicate in separate experiments, and the analysis was performed with Sigma Plot 2001 (SPSS Inc., Chicago, IL). The inhibition mode and kinetic parameters were analysed with Enzyme Kinetics Module 1.1 (SPSS Inc.) equipped with Sigma Plot 2001.