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Determination of free diferulic, disinapic and dicoumaric acids in plants and foods

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ABSTRACT

Hydroxycinnamates are common phenolic compounds of plants and plant foods, often found in substantial quantities. Due to their high *in vitro* antioxidant activity they can easily be oxidized under oxidative conditions. In this study, we found that *in vitro* oxidation of coumaric, ferulic and sinapic acids resulted mainly in dimeric compounds. We hypothesized that these dimers are present in plants and plant foods not only in their bound form but also as free acids that can be extracted from non-hydrolyzed samples. By applying sensitive UHPLC–MS/MS method, we were able to identify and quantify four free hydroxycinnamic acid dimers for the first time, namely 8-8′-disinapic, 8-5′-diferulic, 8-*O*-4′-diferulic and 8-3′-dicoumaric acids, in wheat sprouts, Chinese cabbage, millet sprouts, light beer and parsley. Concentrations of dicinnamates in plant tissues ranged from 0.05 to 2.8 μg g^{-1} DW and the monomer:dimer ratio ranged from 2 to 850.

1. Introduction

Undesirable oxidation and browning of plant food diminishes its quality and could also affect its safety (Friedman, 1996). The oxidation of phenolic compounds is poorly understood at the molecular level and the effects of oxidized phenolic compounds on human health are unknown. Hydroxycinnamic acids, the early metabolites of the phenylpropanoid pathway, are present in substantial quantities in all land plants (Emiliani, Fondi, Fani, & Gribaldo, 2009). Their high *in vitro* antioxidant activity suggests that they are easily oxidized under oxidative conditions. Besides direct oxidation by oxidants and oxidizing radicals, phenolics are commonly oxidized by a few enzymes, including polyphenol oxidase (PPO), peroxidase and laccase (Weng & Chapple, 2010; Yoruk & Marshall, 2003). Whereas PPO oxidizes phenols by adding molecular oxygen to their molecule, class III peroxidases and laccases mediate the one electron oxidation of phenolics, resulting in the formation of radical cations or quinones that are subsequently coupled with other molecules. The products have been suggested to be insoluble polymers (e.g., lignin) or soluble low molecular weight compounds, such as oligomeric proanthocyanidins, lignans and some dimeric alkaloids (Costa et al., 2008). The *in vitro* oxidation and subsequent oligomerization of other highly abundant phenolics, such as quercetin, resveratrol, hydroxycinnamic and hydroxybenzoic acids, have been studied several times, but the oxidation products have not yet been unambiguously identified in plants or have only been identified after release from their insoluble form (Bunzel, 2010; Chervyakovsky et al., 2008; Cichewicz, Kouzi, & Hamann, 2000; Liu, Wan, Huang, & Kong, 2007; Mouterde, Flourat, Cannet, Ducrot, & Allais, 2013; Rouau et al., 2003).

Hydroxycinnamic acids, the products of PAL enzyme, have been demonstrated to protect plant cell against free radicals and oxidative damage (Tamagnone et al., 1998; Yamasaki, Sakihama, & Ikehara, 1997). During the elimination of oxidants, hydroxycinnamic acids as electron donors are oxidized and the majority is irreversibly modified although they can partially be regenerated via the ascorbate and glutathione cycles (Sgherri, Cosi, & Navari-Izzo, 2003). In general, hydroxycinnamic acids are found

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both attached to cell wall and as soluble compounds in food plants. However their oligomers have only been determined after saponification, acid hydrolysis or cinnamoyl esterase treatment (Bunzel, 2010; Faulds, Sancho, & Bartolome, 2002). In the present study, we report that diferulic, disinapic and dicoumaric acids are low molecular weight oxidation products present in common plants and foods as free compounds.

2. Materials and methods

2.1. Chemical synthesis

Sinapic, ferulic and 4-coumaric acids were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). 8-*O*-4′-dehydrodiferulic acid **12b** was isolated earlier (Bunzel, Funk, & Steinhart, 2004). 8,5′-dehydrodiferulic **10b** and 8,3′-dehydrodi-*p*-coumaric **10a** acids were prepared according to published protocols (Ralph, Quideau, Grabber, & Hatfield, 1994; Torres & Rosazza, 2001). Proton and carbon chemical shifts (s) are reported in ppm downfield from internal reference of residual (CHD_2)-$CO(CD_3)$ peak in $(CD_3)_2CO$ (for ^{1}H-NMR; calibrated to 2.05 ppm) and the carbonyl peak in $(CD_3)_2CO$ (for ^{13}C-NMR; calibrated to 206.26 ppm). To synthesize bis-lactone **16c**, a solution of sinapic acid **1c** (1.0 g, 4.46 mmol, 1.0 equiv) in EtOH (5 mL) was added to a solution of dry $FeCl_3$ (1.59 g, 9.81 mmol, 2.2 equiv) in EtOH (12 mL) at RT under vigorous stirring. The reaction was stirred for 2 h, followed by removal of the organic solvents under reduced pressure. The residue was suspended in water (20 mL) and extracted with EtOAc (3 × 50 mL). Combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and the solvents were removed under reduced pressure. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 50:1–>20:1), yielding 665 mg (67%) of slightly yellow crystals. Mp = 232–234 °C; ^{1}H NMR (500 MHz, acetone-d_6) δ [ppm] = 3.79 (s, 12H), 4.07 (d, J = 0.5 Hz, 2H), 5.71 (s, 2H), 6.69 (s, 4H), 7.47 (broad s, 2H); ^{13}C NMR (125 MHz, acetone-d_6) δ [ppm] = 176.7, 149.7, 138.2, 130.5, 104.9, 84.0, 57.4, 49.7. To synthesize lactone (*E*)-**17c**, a solution of dilactone **16c** (150 mg, 0.33 mmol, 1.0 equiv) in THF (1 mL) was cooled to 0 °C and 1.0 M aq. sol. of NaOH (10 mL) was added. The reaction mixture was stirred at 0 °C for 15 min and then acidified to pH 4 (2.0 M aq. HCl). The mixture was extracted with EtOAc (3 × 25 mL) and the resulting organic layers were combined, dried over Na_2SO_4, filtered and the solvents removed under reduced pressure. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 20:1–>10:1), yielding 69 mg (46%) of yellow crystals. Mp = decomp; ^{1}H NMR (500 MHz, acetone-d_6) δ [ppm] = 3.75 (s, 6H), 3.81 (s, 6H), 4.28 (m, 1H), 5.69 (d, J = 2.4 Hz, 1H), 6.63 (s, 2H), 7.02 (s, 2H), 7.33 (broad s, 1H), 7.56 (d, J = 2.0 Hz, 1H), 7.86 (broad s, 1H), 11.2–13.5 (broad s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ [ppm] = 174.5, 171.4, 147.4, 147.2, 141.9, 137.7, 135.2, 130.2, 124.4, 118.1, 107.7, 101.9, 80.5, 56.4, 56.3, 53.5. To synthesize lactone (*Z*)-**17c**, a solution of (*E*)-**17c** (~1.5 mg) in acetone-d_6 (550 μL) was placed in close proximity to a tungsten light and irradiated for 3 days. ^{1}H NMR spectra indicated >98% conversion of (*E*)-**17c** to (*Z*)-**17c**. ^{1}H NMR (500 MHz, acetone-d_6) δ = 3.83 (s, 6H), 3.86 (s, 6H), 4.11 (dd, J = 1.9, 5.0 Hz, 1H), 5.75 (d, J = 5.1 Hz, 1H), 6.77 (s, 2H), 7.19 (d, J = 2.0 Hz, 1H), 7.43 (broad s, 1H), 7.61 (s, 2H), 7.96 (broad s, 1H), 11.2–13.5 (broad s, 1H). To synthesize diacid **14c**, a solution of dilactone **16c** (11 mg, 0.02 mmol, 1.0 equiv) in DMSO (1 mL) was added to 0.1 M phosphate buffer (9 mL, pH 7.4) and the resulting mixture was stirred at RT for 3 days. The water and remaining DMSO were removed by sublimation under high vacuum and the residue was suspended in EtOAc (50 mL). The organic layer was dried over Na_2SO_4, filtered, and the solvents were removed under reduced pressure, yielding the diacid **14c** (46%). ^{1}H NMR (500 MHz, acetone-d_6) δ [ppm] = 3.76 (s, 12H), 7.01 (s, 4H), 7.63 (broad s, 2H), 7.85 (s, 2H), 11.5–13.3 (broad s, 2H); 13C NMR (125 MHz, acetone-d6) δ [ppm] = 56.5, 108.8, 126.4, 126.6, 138.8, 142.6, 148.5, 168.6. To synthesize thomasidioic acid **15c**, a solution of sinapic acid **1c** (500 mg, 2.23 mmol) in water (110 mL, 0.02 M) was gently stirred in an open flask at RT. The pH of the solution was adjusted to 7.5–8.0 using 0.5 M aq. NaOH. Air was bubbled through the solution at RT and the resulting mixture was stirred in the dark for 72 h. The whole mixture was then extracted with CH_2Cl_2 (3 × 150 mL). Combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, filtered and the solvents removed under reduced pressure to yield 378 mg (76%) of thomasidioic acid **15c**. 1H NMR (500 MHz, acetone-d6) δ [ppm] = 3.40 (d, J = 1.5 Hz, 1H), 3.61 (s, 3H), 3.69 (s, 6H), 3.89 (s, 1H), 5.05 (broad s, 1H), 6.35 (s, 2H), 6.91 (s, 1H), 7.68 (s, 1H), 8.01 (broad s, 1H), 8.07 (broad s, 1H), 11.5–13.3 (broad s, 2H); 13C NMR (125 MHz, acetone-d6) δ [ppm] = 40.3, 47.3, 56.55, 56.62, 60.4, 106.2, 108.8, 124.07, 124.11, 125.0, 134.7, 1357, 138.3, 142.6, 146.4, 148.45, 148.51, 168.6, 173.4. Spectral data of the isolated acid **15c** were in agreement with those previously reported (Ahmed, Lehrer, & Stevenso, 1973).

2.2. Oxidation by HRP

Sinapic, ferulic and 4-coumaric acids (1 mM) were oxidized in 35% acetone (in distilled water) by adding hydrogen peroxide (final concentration of 0.5%) and horseradish peroxidases Type II and IV (5–250 μg mL^{-1}; Sigma–Aldrich Fine Chemicals, St. Louis, MO, USA). The reaction was monitored by UPLC–ESI-MS (full scan mode) at various time points (5, 15, 30, 60 and 240 min) after diluting 5 μL of the reaction mixture in 145 μL 10% MeOH.

2.3. Plant and food material

Wheat (*Triticum aestivum*) and millet (*Panicum miliaceum*) sprouts were grown in vermiculite at room temperature and shoots were collected after 4 days. Parsley, Chinese cabbage, radish, cranberries, rice, rice bread, carrot, light beer (lager) and white wine were purchased from a grocery store (Olomouc, Czech Republic).

Table 1
Free dicinnamic acids quantified in plant-derived samples. Concentrations are given as mean ± SD (n = 3).

Compound	MRM	Plant material	Concentration (μg g^{-1} DW)	Monomer/dimer ratio
8-8′-Disinapic acid (**14c**)	401.0 > 357.0	Wheat sprouts	2.30 ± 0.10	2.1
		Chinese cabbage	1.94 ± 0.21	9.9
8-5′-Diferulic acid (**10b**)	385.1 > 341.2	Millet sprouts	0.05 ± 0.01	80.0
8-*O*-4′-diferulic acid (**12b**)	385.1 > 193.1	Light beer	2.78 ± 0.26[a]	852.6
8-3′-Dicoumaric acid (**10a**)	325.1 > 281.2	Parsley	0.15 ± 0.01	133.0

[a] ng mL^{-1}.