Identification of Isorhamnetin 4′-Glucoside in Onions

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Seven major flavonoid compounds in onions were separated, isolated, and identified by Sephadex LH-20 chromatography, high-performance liquid chromatography, thin-layer chromatography, mass spectroscopy, and nuclear magnetic resonance spectroscopy. The flavonoids were quercetin, quercetin monoglucoside, quercetin diglucoside, isorhamnetin, isorhamnetin glycoside, rutin, and kaempferol. Glucosylation of the glucose moiety of isorhamnetin glucoside was the 4′-*O*-glucoside.

Keywords: *Flavonoids; flavonols; flavonol glycoside; quercetin, isorhamnetin 4′-glucoside; onions*

INTRODUCTION

Flavonols and flavonol glycosides are widely distributed in edible fruits and vegetables. Isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone) is a methyl ether of quercetin (3,5,7,3′,4′-pentahydroxyflavone), which is one of the most biologically active and common dietary flavonols. An early report showed that dietary intake of quercetin and its glycosides ranges between 50 and 500 mg per day (Jones and Hughes, 1982), whereas a more recent report indicates the average intake of all flavonoids is 23 mg/day (Hertog et al., 1993). Recently, quercetin has been shown to reduce the carcinogenic activity of several cooked food mutagens, inhibit enzymatic activities associated with several types of tumor cells, enhance the antiproliferative activity of the anticancer agents, and inhibit the growth of transformed tumorigenic cells (Leighton et al., 1992). With these beneficial effects, there has been increasing interest recently in vegetables that contain quercetin and its derivatives. *Allium* vegetables are reported to contain high levels of quercetin and its derivatives (Herrmann, 1976; Hertog et al., 1992; Leighton et al., 1992).

Isorhamnetin was first isolated from the flowers of *Cheiranthus cheiri* (Perkin and Hummel, 1896) and various other plants (Gripenberg, 1962). Omidiji (1990) reported that isorhamnetin showed a strong and increased antimicrobial property in fresh onions when the tissues were damaged and contaminated by other microorganisms. Recently, Leighton et al. (1992) tentatively identified isorhamnetin monoglycoside in onions with no positional specificity of glucosylation. We were able to separate and identify major flavonols and their glycosides in various onion cultivars and identify positional specificity of glucosylation of isorhamnetin glucoside by Sephadex LH-20 chromatography and high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), mass spectroscopy (MS), and nuclear magnetic resonance (NMR) spectroscopy.

MATERIALS AND METHODS

Onions. Seven cultivars of onions [(yellow) Golden, Corona, Copra, Spirit, and Parade; (red) Benny; (white) Sweet] grown in the upstate New York region were harvested in October 1993 and stored at 3 °C and 90% relative humidity (RH) until use. For sampling, ~1 kg of onions was randomly selected, hand peeled, and sliced. The sliced onions (200 g) were immediately frozen at −40 °C and then freeze-dried.

Extraction of Flavonols. Extraction of flavonols was carried out according to the methods of Harborne (1989) and Velioglu and Mazza (1991), with a slight modification. The dried onion slices were blended with 600 mL of a methanol: acetic acid:water (10:1:9) mixture in a Waring blender for 5 min and filtered through Whatman no. 1 filter paper after standing overnight at 4 °C. The filtrate was concentrated with a rotary evaporator to 200 mL. The residue was washed with hexane (3 × 100 mL) in a separatory funnel to remove lipids, carotenoids and other fat soluble materials.

Analysis of Flavonols. The extract (2 mL) was loaded on a C_{18} Sep-Pak cartridge (Waters Associates Company) that was pretreated with 2 mL of methanol and 5 mL of H_2O. The extract was then eluted with 2 mL of a methanol:acetic acid: water (75:5:20) mixture, filtered through a 0.45-μm Millipore filter, and injected onto an analytical HPLC unit (Hewlett-Packard, model 1090M, equipped with a photo diode array detector and a series 300 computer) with an 8 mm × 10 cm C_{18} Radial-Pak column (Waters Associates) and 20-μL sample loop. The column was eluted by a gradient solvent system of 5% acetic acid (80 to 0%) and methanol (20 to 100%) at flow rate of 1 mL/min for 25 min.

Fractionation of Flavonols. The extract (10 mL) was first applied to a C_{18} column (2.5 × 10 cm, 55−105 μm; Waters Associates) to remove sugars and other impurities. The column was pretreated with 50 mL of methanol and then with 50 mL of H_2O. After loading the extract, the column was washed with 40 mL of H_2O and then eluted with 40 mL of methanol. The methanol eluent was dried under reduced pressure, mixed with 2 mL of methanol containing 7.5% glacial acetic acid, and then applied to the Sephadex LH-20 column (2.5 × 60 cm, 25−100 μm, Sigma Chemical Company) according to the method of Leighton et al. (1992). Fractions were eluted with methanol that contained 7.5% acetic acid as a mobile phase at a rate of 1 mL/min. A total of 80 fractions of 10.5 mL were collected. The absorbance was monitored at 280 and 405 nm with a dual path moniter (UV-2; Pharmacia Fine Chemicals). The fractions (each 2 mL) separated by the Sephadex LH-20 column were passed through a 0.45-μm Millipore filter and then injected onto an analytical HPLC unit as above.

Hydrolysis of Flavonols. Acid hydrolysis of quercetin glycosides and TLC analysis of sugar were done according to the method of Harborne (1989) and Markham (1989) with a slight modification. Each flavonol fraction was concentrated to dryness, and then a portion (corresponding to 1−2 mg of flavonol) was dissolved in 2 mL of methanol and mixed with 2 mL of 2 N HCl in methanol for hydrolysis. The aglycon and sugar portions of the flavonols were obtained after hydrolysis at 80 °C for 2 h. The hydrolysate was extracted with ethyl acetate (3 × 10 mL) to separate aglycon, which was identified by HPLC. The aqueous solution containing sugars was

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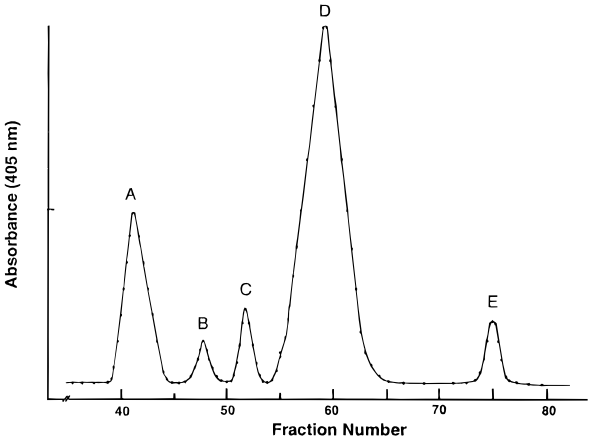

Figure 1. Sephadex LH-20 chromatogram of onion flavonoids. Fractions were eluted with methanol that contained 7.5% acetic acid as a mobile phase at 1 mL/min.

evaporated to dryness, dissolved in water, and then passed through an ion-exchange column (1 × 10 cm, 200−400 mesh, AG2-X8; from Bio-Rad Laboratories) to remove acid. The eluent was evaporated to dryness, dissolved in water, and then applied to a TLC plate (silica gel 60F, 10 × 20 cm, 0.25-mm TLC plate from the Merck Company) and developed with a buthanol:ethanol:water (4:1:2.2) mixture. Sugar spots were developed with a spray reagent of aniline hydrogen phthalate.

Nuclear Magnetic Resonance (NMR). Proton NMR spectra of flavonols dissolved in DMSO-d_6 were obtained with a Varian-NMR (400 MHz).

Fast-Atom Bombardment Mass Spectrometry. Fast atom bombardment (FAB) mass spectra (positive ion mode) were obtained at a resolution of 1000 and acceleration potential of 8 kV on a Fisons VG Analytical (Manchester, England) ZAB-SE double-focusing mass spectrometer. The FAB gun employed xenon at a potential of 8 kV and a current of 1.2 mA. The matrix used was a 3:1 mixture of dithiothreitol and dithioerythritol. A sample of 10 μg in ~3 mL of matrix was used.

RESULTS AND DISCUSSION

Among the reported several extraction solvent systems, 80% ethanol (Bandwein, 1965), 85% methanol (Oleszek et al., 1988), the water and methanol mixture (Harborne, 1989), ethanol:acetic acid:water (Velioglu and Mazza, 1991), and 62.5% methanol (Hertog, 1992), we found that the methanol:acetic acid:water (10:1:9) mixture was the best for all flavonol and its glycosides in onions.

Separation and concentration processes with a C_{18} column and C_{18} Sep-Pak cartridge, respectively, were very useful and efficient (Oleszek et al., 1988). Any amount of residual methanol in the extract interferes with the isolation process, so methanol must be removed completely before the sample passes through the C_{18} column or C_{18} cartridge. A typical elution profile of Sephadex LH-20 chromatogram measured at 405 nm for flavonols and their glycosides in cv. Spirit onions is shown in Figure 1. We were able to separate onion flavonoids into five fractions compared with the four fractions reported by Leighton et al. (1992). The four fractions that correspond to those of Leighton et al. (1992), fractions A, C, D, and E, were identified as quercetin diglucoside, isorhamnetin glycoside, quercetin monoglucoside, and quercetin, respectively. Fraction B was identified as rutin.

An individual fraction obtained from the Sephadex LH-20 was injected onto the HPLC column, and each peak was correlated with those of HPLC from the onion extract. A chromatogram of analytical HPLC for sun-dried onion extract is shown in Figure 2. Five peaks obtained from HPLC were matched with those five peaks of the Sephadex LH-20 chromatogram with HPLC retention times of 9.6, 12.1, 13.2, 14.2, and 15.8 min and corresponded with fractions A, B, D, C, and E, respectively. An additional peak at a retention time (R_t) of 18.3 min was observed during the course of onion storage and identified later as isorhamnetin. When each fraction obtained from the Sephadex LH-20 colum was subjected to acid hydrolysis, and then the hydrolysate was extracted with ethyl acetate and analyzed by HPLC, we obtained a single peak (R_t = 15.8 min) from fractions A, B, and D. The R_t corresponded to that of standard quercetin and the UV−vis spectrum coincided with that of quercetin. Fraction C produced one peak on HPLC after hydrolysis that had an R_t of 18.3 min. When the whole onion extract was hydrolyzed and then analyzed by HPLC, we obtained one major peak and two minor peaks, as shown in Figure 3. The first peak (R_t = 15.8 min) matched with the R_t and spectrum of the standard quercetin, the second peak (R_t = 17.8 min) matched with the R_t and spectrum of standard kaempferol, and the third peak (R_t = 18.3 min) matched with the spectrum of isorhamnetin (Mabry et al., 1970). Analysis of sugars by TLC showed that glucose was present in all hydrolysates of the four fractions A, B, C, and D from the Sephadex LH-20 column.

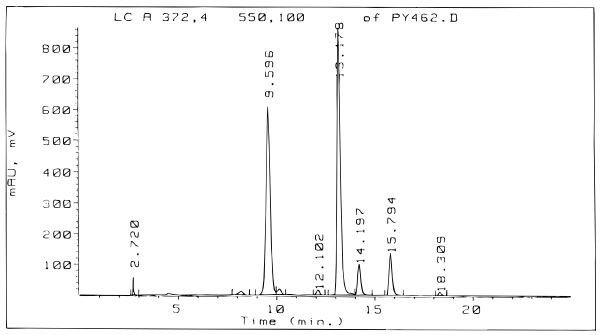

Figure 2. Chromatogram of analytical HPLC for sun-dried onion flavonoids: R_t = 9.5, quercetin diglucoside; R_t = 12.1, rutin; R_t = 13.1, quercetin monoglucoside; R_t = 14.1, isorhamnetin 4′-glucoside; R_t = 15.7, quercetin; R_t = 18.3, isorhamnetin.

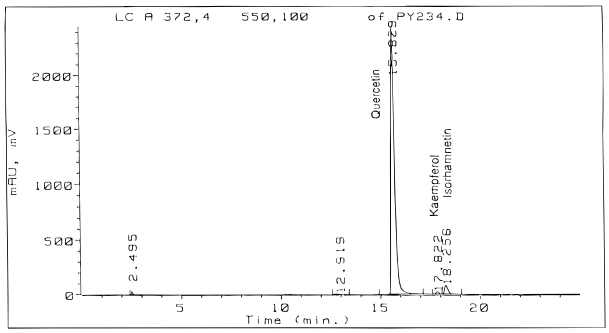

Figure 3. Chromatogram of analytical HPLC for onion flavonoids after acid hydrolysis: R_t = 15.8, quercetin; R_t = 17.8, kaempferol; R_t = 18.2, isorhamnetin.

FAB-MS of fraction C in the positive-ion-mode produced a spectra containing main ions at m/z 479, corresponding to the $(M + H)^+$ ion of isorhamnetin glucoside, and m/z 317, corresponding to the $(M -$ glucose$)^+$. The compound that appeared in the HPLC (R_t = 18.3 min) after acid hydrolysis showed a spectrum at m/z 317, corresponding to the $(M + H)^+$ of isorhamnetin.

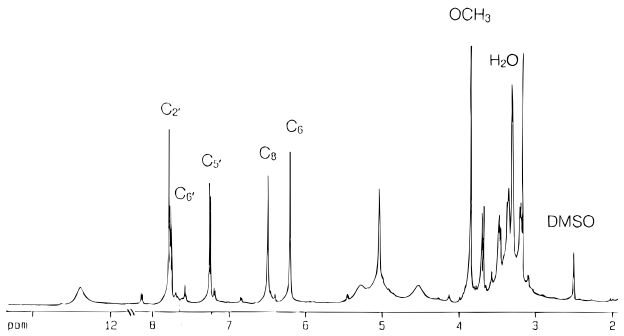

Figure 4. ^{1}H NMR spectrum of isorhamnetin 4′-glucoside.

The proton NMR data of the compound that appeared in HPLC spectrum, with the R_t of 18.3 min after acid hydrolysis, are as follows: δ 3.83*s* (3H, s, MeO-3′), 6.19 (1H, *d*, J = 1.4 Hz, H-6), 6.47 (1H, *d*, J = 1.4 Hz, H-8), 6.93 (1H, *d*, J = 8.5 Hz, H-5′), 7.68 (1H, *dd*, J = 1.3, 8.5 Hz, H-6′), 7.74 (1H, *d*, J = 1.3 Hz, H-2′), 9.82 (1H, *s*, OH), 10.82 (1H, *s*, OH), and 12.45 (1H, *s*, OH-5). These data are consistent with the suggestion that the compound is isorhamnetin (Mabry et al., 1970; Markham, 1994). The proton NMR data (Figure 4) of fraction C (or HPLC R_t = 14.2) are as follows: δ 3.80s (3H, *s*, MeOH-3′), 6.20 (1H, *d*, J = 2.2 Hz, H-6), 6.50 (1H, *d*, J = 2.2 Hz, H-8), 7.25 (1H, *d*, J = 8.8 Hz, H-5′), 7.77 (1H, *dd*, J = 2.4, 8.8 Hz, H-6′), 7.79 (1H, *d*, J = 2.4 Hz, H-2′), 9.43 (1H, *s*, OH), 9.73 (1H, *s*, OH), 10.76 (1H, *s*, OH), and 12.43 (1H, *s*, OH-5). These data are consistent with isorhmnetin 4′-glucoside.

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