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Glucosinolate accumulation in three important radish (*Raphanus sativus*) cultivars

Sun-Ju Kim[1], Md Romij Uddin[2,*] and Sang Un Park[2,*]

[1]Department of Bio Environmental Chemistry, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon, 305-764, Korea
[2]Department of Crop Science, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon, 305-764, Korea

***Corresponding authors: supark@cnu.ac.kr**

Abstract

Radish, *Raphanus sativus*, is an important dietary vegetable in Asian countries, especially in China, Japan, and Korea. In this study, the variation of glucosinolate (GSL) contents among three radish cultivars, including Seo Ho, Man Tang Hong, and Hong Feng No. 1 were evaluated. While significantly different levels of 13 GSLs were observed in both the skin and flesh of these radish cultivars, the levels in the skin, in general, were higher than those in the flesh. Among these 13 GSLs, glucoraphanin, glucobrassicin, methoxyglucobrassicin, and glucoraphasatin were present in both the skin and flesh of all 3 cultivars studied. Particularly, a large amount of glucoraphasatin was detected in the skin of all cultivars. Glucoraphenin was dominant in the flesh of Man Tang Hong, as well as in the skin of Hong Feng No. 1. Moreover, the skin of Man Tang Hong contained the highest level of glucobrassicin, which was 5.1, 4.6, and 4.5 times higher than the level in the flesh of Man Tang Hong, Hong Feng No. 1, and Seo Ho, respectively. In addition, the variation among the glucobrassicin levels in the skin of 3 cultivars was smaller than that among their flesh. Furthermore, neoglucobrassicin was present only in the skin of Seo Ho. Our results suggest that colorful radish (skin and flesh) cultivars might play a vital role for getting health benefit glucosinolate through consumption as vegetables.

Keywords: Flesh; Glucosinolates; HPLC-ESI-MS/MS; *Raphanus sativus*; Skin.
Abbreviation: ANOVA_Analysis of variance, DS-GSL_Desulfo-glucosinolates, ESI-MS_Electrospray ionization -mass spectrometer, GSL_Glucosinolate, HPLC_High Performance Liquid Chromatography, RDA_Rural Development Administration, SAS_Statistical Analysis Systems.

Introduction

Members of the Brassicaceae family are among the most commonly grown vegetables worldwide and have been shown to contain very potent phytochemicals, glucosinolates (GLSs), and their breakdown products (Mithen et al., 2000). GLSs, which are b-thioglucoside-N-hydroxysulfates (cis-N-hydroximinosulfate esters), are sulfur-rich, anionic secondary metabolites derived from glucose and an amino acid. They can be found almost exclusively in plants of the order Brassicales (including the family Brassicaceae, Capparidaceae, and Caricaceae), but also in the genus *Drypetes* (family Euphorbiaceae). To date, about 200 different glucosinolates are known to occur naturally in plants (Rodman et al., 1996; Clarke, 2010). GLSs are hydrolyzed into several bioactive compounds by the endogenous enzyme myrosinase (β-thioglucosidase glucohydrolase; EC 3.2.3.1). The hydrolytic products include substituted isothiocyanates, nitriles, thiocyanates, epithionitriles, and oxazolidinethiones, all of which not only serve as defense for the plant in nature but also exhibit various biological activities possibly beneficial for human health (Gil and MacLoed, 1980; Fenwick et al., 1983; Al-Gendy et al., 2010; Björkman et al., 2011). Moreover, GLSs from vegetables of the Cruciferae family have been associated with a reduced risk of cancers of the lung, stomach, breast, prostate, pancreas, colon, and rectum (Herr and Büchler, 2010; Krzyzanowska et al., 2011). Radish (*Raphanus sativus*), which belongs to the Brassicaceae family, is an edible root vegetable cultivated and consumed worldwide. Radish varieties differ in size, color, and cultivation requirements (Gutierrez and Perez, 2004; Hara et al., 2009). The number of documented cases of GLS analysis from different parts of radish remains small and to our knowledge, no previous report or publication has established for the GLS analysis either in the skin or flesh of different cultivars of radish. Therefore, this study was conducted to compare the levels of GLS content in skin and flesh of 3 radish cultivars (including Seo Ho, Man Tang Hong, and Hong Feng No. 1), where GLS compounds were extracted from the radish skin or flesh and then quantified.

Results and Discussion

Identification of GLS in the skin and flesh of radish cultivars

Different parts (skin and flesh) of 3 radish cultivars were analyzed, and 13 different GSLs including 1 unknown GSL were detected in both the skin and flesh (Figure 1). Specifically, the skin and flesh of Seo Ho contained 10 and 9 GSLs,respectively, whereas the skin and flesh of Man Tang Hong contained 8 GSLs and the skin and flesh of Hong Feng No. 1 contained 10 and 8 GSLs, respectively (Table 1).

Table 1. Glucosinolates (GSLs) identified in the skin and flesh of radish (*Raphanus sativus*) cultivars.

No.[a]	RT[b] (min)	Trivial names	Structure of R-groups	Semisystematic names of R-groups	Parental amino acids	Compound groups	Molecular weight[c] (m/z) $[M+H]^+$	Response factor[d]
1	6.12	Glucoraphanin	CH_3-SO-$(CH_2)_4$-	4-Methylsulfinylbutyl	Methionine	Aliphatic	358	1.07
2	8.78	Glucoalyssin	CH_3-SO-$(CH_2)_5$-	5-Methylsufinylpenyl	Methionine	Aliphatic	372	1.07
3	9.59	Gluconapoleiferin	CH_2=CH_2-CH_2-CHOH-CH_2	2-Hydoxy-4-pentenyl	Methionine	Aliphatic	324	1.00
4	12.51	Glucoraphenin	C_6H_5-OH-(CH_2)-	4-Hydroxybenzyl	Methionine	Aliphatic	345	1.00[e]
5	14.69	Gluconapin	CH_2=CH-(CH_2)-	3-Butenyl	Methionine	Aliphatic	372	1.11
6	16.26	Glucobrassicanapin	CH_2=CH_2-$(CH_2)_3$-	4-Pentenyl	Methionine	Aliphatic	308	1.15
7	17.93	4-Hydroxyglucobrassicin	4-OH-indole-3-CH_2	4-Hydroxy-3-indolylmethyl	Tryptophan	Indolyl	385	0.28
8	20.77	Glucoerucin	CH_3-S-$(CH_2)_4$	4-Methylthiobutyl	Methionine	Aliphatic	342	1.00[e]
9	21.34	Unknown	Unknown	ND	ND	ND	ND	1.00[e]
10	22.16	Glucoraphasatin	CH_3-S-CH==CH-$(CH_2)_2$	4-Methylthio-3-butenyl	Methionine	Aliphatic	340	1.00[e]
11	22.80	Glucobrassicin	Indole-3-CH_2-	3-Indolylmethyl	Tryptophan	Indolyl	369	0.29
12	25.26	4-Methoxyglucobrassicin	Indole-4-OCH_3-	4-Methoxy-3-indolylmethyl	Tryptophan	Indolyl	399	0.25
13	31.22	Neoglucobrassicin	Indole-3-OCH_3-	N-Methoxy-3-indolymethyl	Tryptophan	Indolyl	399	0.20

[a]No., the elution order of glcosinolates from HPLC chromatogram (Figure 1). [b]RT, retension time. [C] as a desulfo-GSLs. [d]The international organization for standardization (ISO 9167-1, 1992. [e]Undecided by the ISO. ND, not determined.

Table 2. Glucosinolate levels (mg/g dry wt.) in three radish cultivars (n = 3).

No.[a]	Glucosinolate	Radish cultivars					
		SH[b]-Skin	SH-Flesh	MTH[b]-Skin	MTH-Flesh	HF[b]-Skin	HF-Flesh
1	Glucoraphanin	1.51 b[c]	1.27 bc	1.31 b	1.01 d	1.74 c	1.43 b
2	Glucoalyssin	0.43 b	0.37 c	0.57 b	ND	0.89 c	ND
3	Gluconapoleiferin	0.73 b	0.74 bc	ND	0.73 d	ND	1.77 b
4	Glucoraphenin[z]	ND[d]	ND	0.78 b	27.92 a	12.02 b	ND
5	Gluconapin	ND	ND	ND	1.75 cd	0.67 c	ND
6	Glucobrassicanapin	1.53 b	5.31 b	ND	ND	ND	11.80 b
7	4-OH-glucobrassicin[z]	ND	ND	ND	5.06 c	3.04 c	ND
8	Glucoerucin	1.30 b	0.64 bc	0.60 b	ND	2.04 c	0.71 b
9	Unknown-3	1.86 b	0.63 bc	0.60 b	ND	1.35 c	0.46 b
10	Glucoraphasatin	155.62 a	59.68 a	53.58 b	16.51 b	116.0 a	55.14 a
11	Glucobrassicin	3.42 b	1.28 bc	5.70 b	1.11 d	5.26 c	1.24 b
12	4-Methoxyglucobrassicin	2.06 b	1.18 bc	3.17 b	2.41 cd	2.10 c	0.80 b
13	Neoglucobrassicin	0.21 b	ND	ND	ND	ND	ND
Total		**168.44±59.95**	**71.09±7.38**	**65.66±29.41**	**56.51±8.96**	**144.87±11.48**	**73.35±32.42**

[a]No. corresponds to the elution order by the HPLC analysis in Fig. 1. [b]Abbreviations: SH, Seo Ho; MTH, Man Tang Hong; HF, Hong Feng No.1. [c] Mean values (mean of three replicates with three samples from each replicate) indicated by the same letter in a column do not differ significantly at 5% level (Duncan Multiple Range Test). [d]ND, not detected.

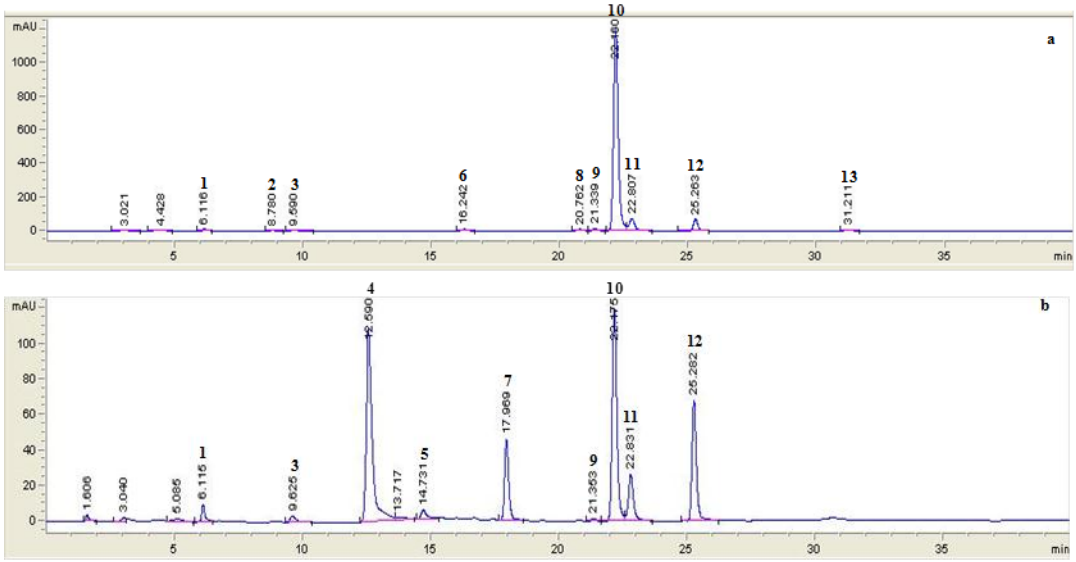

Fig 1. HPLC chromatograms of glucosinolates[1-13] in the skin of radish cultivars, including Seo Ho (a) and Hong Feng No.1 (b). [1]Glucoraphanin, [2]Glucoalyssin, [3]Gluconapoleiferin, [4]Glucoraphenin, [5]Gluconapin, [6]Glucobrassicanapin, [7]4-Hydroxyglucobrassicin, [8]Glucoerucin, [9]Unknown, [10] Glucoraphasatin, [11]Glucobrassicin, [12]4-Methoxyglucobrassicin, [13]Neoglucobrassicin.

Variation of GLS content in different parts of radish cultivars

Our data show that the GSL levels in the skin, in general, were higher than those in the flesh for all 3 radish cultivars tested. Moreover, among these 13 GSLs, glucoraphanin, glucobrassicin, 4-methoxyglucobrassicin, and glucoraphasatin were present in all the skin and flesh. Particularly, a large amount of glucoraphasatin was contained in all 3 radish cultivars, with approximately 3 times more in the skin than in the flesh. In addition, the highest level of glucoraphasatin was found in the skin of Seo Ho, with the second highest in the skin of Hong Feng No. 1. Glucoraphenin was dominant in the flesh of Man Tang Hong and the skin of Hong Feng No. 1, whereas no glucoraphenin was found in either skin or flesh of Seo Ho. 4-OH-glucobrassicin was only present in the flesh of Man Tang Hong and the skin of Hong Feng No. 1. The skin of Man Tang Hong contained the highest level of glucobrassicin, which was 5.1, 4.6, and 4.5 times higher than the levels in the flesh of Man Tang Hong, Hong Feng No. 1, and Seo Ho, respectively. We also observed that the variation among the glucobrassicin levels in the skin of 3 cultivars was smaller than that in the flesh. The highest 4-methoxyglucobrassicin level was observed in the skin of Seo Ho, which was 4, 2.7, and 1.3 times higher than the levels in the flesh of Hong Feng No. 1, Seo Ho, and Man Tang Hong, respectively. The skin and flesh of Hong Feng No.1 contained the highest glucoraphanin content, followed by Seo Ho and Man Tang Hong. Glucobrassicanapin was present in both the skin and flesh of Seo Ho, as well as in the flesh of Hong Feng No. 1. The glucobrassicanapin content was the highest in the flesh of Hong Feng No. 1, which was 7.7 and 2.2 times higher than the levels in the skin and flesh of Seo Ho, respectively. Gluconapin was present only in the flesh of Man Tang Hong and the skin of Hong Feng No. 1; the content in the former was 2.6 times higher than that in the latter. No glucoalyssin was present in the flesh of Man Tang Hong or Hong Feng No. 1. The glucoalyssin level in the skin of Seo Ho was higher, compared to its flesh, and the highest glucoalyssin level was found in the skin of Hong Feng No. 1. Glucoerucin was present in all the radish cultivars, except for the flesh of Man Tang Hong. The glucoerucin content was the highest in the skin of Hong Feng No. 1, with the second highest in the skin of Seo Ho, whereas the level was the lowest in the skin of Man Tang Hong. Neoglucobrassicin was present only in the skin of Seo Ho. Many GSLs and their derived products have been reported to possess a variety of pharmacological and toxic activities, including goitrogenic activity, anti-bacterial and anti-fungal actions, and the ability to reduce the risk of certain human cancers (Zhang and Talalay, 1994; Talalay and Zhang, 1996). The role of sulforaphane was studied in depth by Talalay's research group in the 1990's because of its well-known activity as a potential cancer chemopreventive agent (Zhang et al., 1992; Talalay, 1998). Sulforaphane is hydrolyzed from glucoraphanin, which is an abundant GSL in the Brassica species. Talalay's research group identified lines/cultivars and developmental stages of broccoli with higher glucoraphanin content (Fahey et al., 1997; Yanaka et al., 2009), later named "Super sprouts". In the Brassica genus, aliphatic GSLs are most common, but traces of indolic GSLs are also present in Brassica species (Brown et al., 2002; Kumar and Andy, 2012). In the present study, the highest GSL produced was neoglucobrassicin, which ranged from 41 to 70 μmol/g dry wt., except for the control (1/2 MS) (Table 1). These data are in agreement with data from Spanish broccolis (12 ~ 71 μmol/g dry wt.) grown under various climatic and fertilization conditions in an experimental field (Vallejo et al., 2003). It is well-known that the GSL content in plants varies widely according to environmental effects such as agronomic management, climatic conditions and mineral nutrient availability, different plant organs, developmental stage, and varieties (Brown et al., 2002; Kumar and Andy, 2012. Li et al (2008) reported that remarkable differences in glucosinolate profiles in sprouts,leaves and edible roots of radish were observed. The contents of total GLSs in the sprouts and edible roots were higher than that in the leaves. The major GS in edible roots and sprouts was 4-methylthio-3-butenyl GLS, accounting for 75.5% and 71.5% of the total GSs, respectively. Indol-3-ylmethyl GLS was the major GLS in radish leaves, accounting for 57.1% of the total leaf GSs. The findings of Li et al (2008) are in agreement with our findings from this study as the content of GLS varied among the cultivars and also the

different parts of radish cultivars.

Materials and methods

Plant materials

Roots of 3 colorful radish cultivars, including Seo Ho, Man Tang Hong, and Hong Feng No. 1, were acquired from the Rural Development Administration (RDA; Suwon, Korea). Plants were grown in a greenhouse and harvested at 14 to 18 weeks after sowing at the experimental farm of RDA in 2009. The colors of the skin and flesh of Seo Ho were white, whereas Man Tang Hong had white and green colored skin and red interior flesh, and Hong Feng No. 1 had red colored skin and white interior flesh at maturity. Prior to experiments, radish roots were manually peeled, and the epidermal tissues (skin) and flesh were cut into small cubes. Samples were then freeze-dried at −80°C for at least 48 h, ground to a fine powder using a mortar and pestle, and stored in a refrigerator until the high performance liquid chromatography (HPLC) analysis.

Chemicals

HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA), and sodium acetate ($NaC_2H_3O_2{\cdot}3H_2O$) was obtained from Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan). Aryl sulfatase (type H-1, EC 3.1.6.1), sinigrin (2-propenyl GSL), and DEAE-Sephadex A-25 were supplied by Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Ultra-pure water used in this study was generated by the PURELAB Option-Q System (ELGA Lab Water, VWS Ltd., UK).

Extraction of desulfo-glucosinolates (DS-GSLs)

DS-GSLs were extracted according to the procedure described by Kim et al. (2007) and ISO 9167-1 (1992). Briefly, crude GSLs were extracted from lyophilized powder (100 mg) with 70% (v/v) boiling methanol (1.5 mL) in a water bath at 70°C for 5 min to inactivate endo-myrosinases. Mixtures were centrifuged at 12,000 × *g* at 4°C for 10 min in a Hanil microcentrifuge (Micro 17R, Incheon, Korea), and resulting supernatants were collected into 5-mL test tubes. The residue was re-extracted twice in the same manner, and supernatants were combined and considered as crude GSL extracts. Extracts were loaded using 1,000-µL pipet tips into a mini-column previously packed with DEAE-Sephadex A-25 (H^+ form by 0.5 M sodium acetate, approximately 40 mg dry wt.). After proper washes with ultrapure water, GSLs were desulfated by adding an aryl sulfatase solution (75 µL) to the column. After overnight incubation (16–18 h) at ambient temperature, DS-GSLs were eluted with 0.5 mL (× 3) of ultrapure water into 2-mL microcentrifuge tubes. Eluates were then filtered through a 0.45-µm hydrophilic PTFE syringe filter (Ø, 13 mm; Advantec, Tokyo, Japan) into brown HPLC vials and immediately stored in a refrigerator at 4°C until the HPLC analysis.

HPLC analysis of DS-GSLs

The separation of DS-GSLs was carried out on a reversed-phase Inertsil ODS-3 column (150 × 3.0 mm i.d. with a particle size of 3 µm; GL Sciences, Tokyo, Japan) with an E type cartridge guard column (10 × 2.0 mm i.d., 5 µm) using an Agilent Technologies 1200 series (Palo Alto, CA, USA). The column oven temperature and the detection wavelength were set at 40°C and 227 nm, respectively, and the flow rate was 0.2 mL/min. The mobile phase consisted of ultrapure water (solvent A) and CH_3CN (solvent B). The gradient programs were as follows: a linear step from 7% to 24% of solvent B for 18 min, 24% of solvent B for the next 14 min, followed by a quick drop down to 7% B at 32.1 min and isocratic conditions with 7% B for 8 min (40 min total). Individual GSLs were identified based on retention time of HPLC following our previous published report (Kim et al., 2007), and quantified with a 5 ml of external standard sinigrin solution (0.1 mg/ml), which was passed following the same extraction process, with its HPLC area and response factor (ISO 9167-1, 1992). All DS-GSLs were referred to as GSLs in the present study, even though they were desulfated.

Qualitative LC/ESI-MS analysis of DS-GSLs

Mass spectrometry (MS) data were acquired by using an electrospray ionization (ESI)-mass spectrometer of an API 4000 Q TRAP system (Applied Biosystems, Foster City, CA, USA) in a positive ion mode ($[M+H]^+$), equipped with an Agilent 1200 series HPLC. Analytical MS conditions were as follows: scan range and time, *m/z* 100–800 and 4.8 s, respectively; curtain gas (N_2), 20 psi; heating gas temperature, 550°C; nebulizing and heating gas, 50 psi; ion spray voltage, 5,500 V; and declustering and entrance potential, 100 V and 10 V, respectively.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) with sums of squares partitioned to reflect trial effects using the SAS Software release 9.2 and means were separated via Duncan Multiple Range Test. The levels of GSLs were higher in the skin than those in the flesh of radish cultivars. Especially, the skin of Man Tang Hong contained the highest level of glucobrassicin, which was 5.1, 4.6, and 4.5 times higher than the level in the flesh of Man Tang Hong, Hong Feng No. 1, and Seo Ho, respectively. Therefore, findings of this study suggest that colorful radish (skin and flesh) cultivars might play a vital role for getting health benefit glucosinolate through consumption as vegetables. Our current laboratory efforts are aimed at further improving glucosinolate compound production in different vegetables crop.

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