ORIGINAL ARTICLE

Bioactive glucosinolates and antioxidant properties of broccoli seeds cultivated in Thailand

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Abstract

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One of the most significant health concerns of cruciferous vegetables is the presence of biologically active compounds, glucosinolates. Broccoli (*Brassica oleracea* var. *italica*) is a nutritionally important crop grown all over the world. Glucosinolates have been found to have anti-cancer properties. The primary purpose of this study was to evaluate glucosinolate content and antioxidant property in the seeds of broccoli cultivars widely grown in Thailand. Glucosinolates were analyzed with high performance liquid chromatography (HPLC). Total and individual glucosinolate levels varied significantly among cultivars. In all broccoli seeds, 4-methylsulfinylbutylglucosinolate (glucoraphanin) was the predominant glucosinolate. The highest total glucosinolates was 65.5 μmol/g DW in 'Top Green #067' cultivar, followed by 'Packman' (58.6), 'Green Queen' (51.2), 'Pak Ging' (25.5) and 'Rod Fai' (20.3). The antioxidant capacities, including ABTS radical scavenging activity and ferrous ion chelating ability in the methanol and water extracts, were found to be high.

Key words : ABTS assay, broccoli seeds , cultivar, FRAP assay, glucosinolates

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The presence of xenobiotics and/or the absence of some essential nutrients in our diet can produce or enhance a certain susceptibility to illness. Nutraceutical and functional food ingredients have been popularly used in health products. The most important nutraceutical formulations contain vitamins and minerals in combination with antioxidant(s) such as carotenoids, omega-3 fatty acids, glucosinolates and/or degradants in the finished products (Palmieri, 2000). Glucosinolates, a class of plant secondary metabolite, are β-thioglucoside *N*-hydroxysulfates [also known as (Z)-(or *cis*)-*N*-hydroximinosulfate esters or *S*-glucopyranosyl thiohydroximates], with a side chain (R) and a sulfur-linked β-D-glucopyranose moiety. The common structural feature of intact glucosinolates is shown in Figure 1 (Fahey *et al*., 2001). Glucosinolates and/or their breakdown products have long been known for their fungicidal, bacteriocidal, nematocidal, allelopathic properties (intercrops or forecrops for weed control) and have attracted intense research interest because of their cancer chemoprotective attributes (Fahey *et al*., 2001; Verhoeven *et al*., 1997). Glucosinolates are found in dicot plants, particularly in the Order Capparales, comprising the Capparaceae, Brassicaceae (Cruciferae), Loeberliniaceae, Moringaceae, Rasedaceae and Tovariaceae. Brassicaceae (Cruciferae) is one of the 10 most economically important plant families and includes vegetables such as broccoli, brussels sprouts, cabbage, cauliflower, kale, radish and various mustards. Glucosinolate content in some tissues of the *Brassica* vegetables is approximately 1% of dry weight (DW), although the content has been found to be as high as 10% in the seeds of some plants. Among the cultivated Brassicaceae, broccoli attracted attention after the discovery that it contains high levels of isothiocyanate sulforaphane [4-(methylsulfinyl) butyl isothiocyanate], produced from the breakdown of the glucosinolate glucoraphanin [4-(methylsulfinyl) butyl glucosinolate] and showed great potential anticarcinogenic properties. Sulforaphane is the most potent naturally occurring Phase II enzyme inducer and considered to play an important role in the detoxification of xenobiotic compounds when assimilated with diet (Fahey *et al*., 2001; Rosa *et al*., 1997).

Broccoli grows well in most types of soil, but rather prefers those crumbling or friable soils with pH around 6.0-6.5, high soil humidity, and sunlight. It develops best during cool seasons. The optimal temperatures are between 18-27ºC, but preferably at the average temperature of 20ºC (George, 1999). In Thailand, the most suitable period is between November and December. There are many types of F1 hybrid broccoli cultivars in Thailand. F1 hybrid cultivars are the results of crossing two inbred lines, which have been maintained under strict control, or under the supervision of commercial plant breeders, which are known to produce a desirable hybrid. The advantages of F1 hybrid broccoli cultivars include uniformity, increased vigor, earliness, higher yield and resistance to specific pests and pathogens. This research focused specifically on the determination of glucosinolate content and antioxidant properties in the seeds of various Thai broccoli cultivars.

Materials and Methods

Seed materials

Seeds from five broccoli cultivars, which included 'Green Queen', 'Packman', 'Pak Ging',

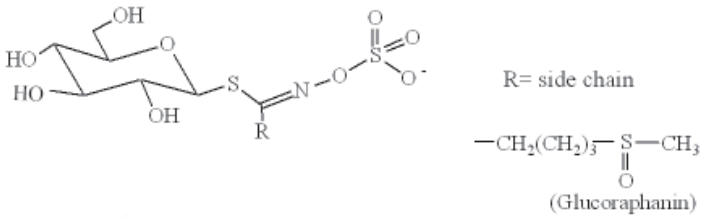

Figure 1. General structure of intact glucosinolates

'Rod Fai', and 'Top Green #067' obtained from Phu Ruea Highland Cultivation Experimental Station, Loei, Thailand, were used in the study. The seeds were harvested in May 2003, and were sealed in plastic-laminated aluminum sachets. The sachets were stored at 4ºC until use. Broccoli seeds were sown directly in seedbeds or containers. Young plants were planted out into the field when they had reached the 5-7 true leaf stage. Under favorable environmental conditions, it took 60-150 d for broccoli, depending on cultivars, to grow from seedling to harvest.

Chemicals and equipment

ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt was obtained from Sigma-Aldrich Chemical Co., St. Louis. Ascorbic acid was purchased from Fisher, UK, and other chemicals were potassium persulfate (Carlo Erba Reagenti, Italy), Trolox (Fluka, Switzerland), quercetin dihydrate (Arcos organics, Belgium), and iron (III) chloride heptahydrate (BDH, Englnd). Sulfatase, DEAE Sephadex A25, benzylglucosinolate and TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) were purchased from Sigma Chemical Co., St. Louis. Certified rapeseed reference materials BCR-367R was obtained from Commission of the European Community Bureau of References (Brussels, Belgium). All other chemicals and solvents were obtained from Sigma Chemical Co., St. Louis, or J.T Baker, Phillipsburg, NJ.

The HPLC system was equipped with a Hewlett Packard Model HP 1090 Photodiode Array Detector, and an automated injector. Data were processed with HP ChemStation software.

Glucosinolate assay

The AOCS methods (AOCS, 1993) were modified for glucosinolate analysis. Broccoli seeds were homogenized with a mortar and pestle. A 0.2 g of ground sample from each replicate was placed in a capped 15 mL glass tube and heated on a heating block at 75ºC for 1 min. Warm methanol (2 mL) was then added to the test tube followed by 0.25 mL of benzylglucosinolate (1 mg/mL) as an internal standard. The tube was continuously heated for 10 min and centrifuged at 3000g for 3 min. After centrifugation, the supernatant was transferred to a 10 mL flask. The residue was extracted twice with 2 mL of warm methanol and the solution was centrifuged each time. The supernatant was collected, combined with the previously saved supernatant, and mixed thoroughly in the same flask. One mL of the supernatant was transferred to DEAE Sephadex A-25 column and allowed to drain and desulfoglucosinolates were obtained after the treatment of the column with sulfatase (500 µL). The column was capped for 12 h. Desulfated glucosinolates were eluted off the column with three 1 mL portion of water and separated by HPLC on an analytical 5 µLichrosorb Hibar RP-18 column (Merck, Darmstat, Germany) with a mobile phase of acetonitrile (50%) plus deionized water (50%) containing 5 mM tetraoctylammonium bromide (solvent A) and acetonitrile (solvent B). The flow rate was 0.20 mL/min at 229 nm wavelength. The following gradient elution was used for separation: 0%B at 0 min, 100%B at 23 min. Solvents were filtered (0.45 µm) and degassed for 30 min before use. Individual glucosinolates were identified by comparison of retention times with those of known reference compounds and by adding separate, individual, pure compounds to broccoli seeds extracts and observing the rise in peak height. For data analysis, analyses of variance (ANOVA) were performed using a general linear model test for each cultivar. Mean separation was determined by least significant differences (Fisher's LSD) at P=0.05.

Antioxidant assay

Preparation of sample extracts

Broccoli seeds were milled and, in turn, extracted with five solvents: chloroform, ethyl acetate, ethanol (70%), methanol and water. Two-step extraction was applied by shaking flasks with 10±0.01 g of seed and 100 mL (50+50) of solvent in a shaking machine (Shaking Water Bath, model SB-200-10, Thailand). Each extraction step was completed in 3 h. The extracts were filtered, concentrated in a rotary evaporator (Buchi, Flawil,

Switzerland) at approximately 40ºC and tested for antioxidant activities.

I) ABTS radical cation decolourisation assay

ABTS radical cation decolourisation test (Re *et al.*, 1999) is also a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. ABTS radical was generated by oxidation of ABTS with potassium persulfate. Three mL of $ABTS^{o+}$ cation solution were mixed with 30 mL seed extract solution in 1 cm path length disposable microcuvette. The absorbance at 734 nm was determined after reaction for 4 min. The lower the absorbance the stronger the reducing power compared with three known antioxidants. The antioxidants included ascorbic acid as a vitamin widely found in fruits and vegetables, quercertin representing the group of polyphenols, and Trolox as water-soluble vitamin E analogue. The percentage of ABTS scavenging activity is expressed by [1-(test sample absorbance/ blank sample absorbance)] x 100.

II) Ferric reducing antioxidant power (FRAP assay)

The FRAP assay as described by Benzie and Strain (1996) was used with minor modifications. A 50 mL seeds extraction solution and 30 mL water were pipetted into a 1 cm path length disposable microcuvette. A 200 mL FRAP solution (300 mM acetate buffer pH 3.6: 10 mM TPTZ in 40 mM HCl: 20 mM $FeCl_3$; 10:1:1) was added, mixed for 10 s and the absorbance at 593 nm was taken after 4 min. The higher the ferrous ion chelating ability of the test sample the lower the absorbance. The percentage of ferrous ion chelating ability is expressed by [1-(test sample absorbance/ blank sample absorbance)] x 100.

Results and Discussion

Content of bioactive glucosinolates in broccoli seeds

At least seven glucosinolates were detected in the seeds, which were classified into three categories: (1) methylsulfinylalkyl glucosinolates (glucoraphanin, glucoiberin, glucoalyssin); (2) methylthioalkyl glucosinolates (glucoerucin, glucoberverin, glucoalyssin); and (3) indole glucosinolates (4-hydroxyglucobrassicin, neoglucobrassicin). Total and individual glucosinolates levels varied significantly among cultivars. The compound 4-methylsulfinylbutyl glucosinolate (glucoraphanin) was the predominant glucosinolate in broccoli seeds. Total glucosinolates were significantly higher in 'Top Green #067' (64.5 µmol/g DW). Those of other cultivars are, in descending order, as follow: 'Green Queen' (51.2 µmol/g DW), 'Packman' (44.3 µmol/g DW), 'Pak Ging' (25.5 µmol/g DW) and 'Rod Fai' (20.3 µmol/g DW). All five cultivars were planted in the same environment and there was no special treatment to the soil bases. A typical glucosinolate chromatogram from broccoli seeds is shown in Figure 2.

The glucosinolate pattern of all cultivars was similar to that described by other authors (Kushad *et al.*, 1999; Rosa *et al.*, 1997; Rosa and Rodrigues, 2001). Table 1 shows the amount of the total and main individual glucosinolate expressed in term of µmol/g DW of the seeds. 'Top Green #067' contained the highest level of total and individual glucosinolates, with glucoraphanin at 48.9 µmol/g DW accounting for 75.8% of the glucosinolates. The compounds 4-methylsulfinylbutyl-, 4-methoxyindol-3-ylmethyl, 4-methylthiobutyl and 4-hydroxy-3-indolylmethyl glucosinolate were common to all cultivars. Only 1-methoxyindol-3-ylmethyl could not be detected in 'Rod Fai' cultivar. Glucoraphanin concentrations in broccoli seeds ranged from 11.4 to 48.9 µmol/g DW, while neoglucobrassicin ranged from 0.0 to 0.2 µmol/g DW. Glucoerucin concentration was lowest in 'Pak Ging' (5.2 µmol/g DW) and highest in 'Top Green' (11.0 µmol/g DW), which was slightly higher than 'Packman' (10.2 µmol/g DW). The concentration of 4-methoxyglucobrassicin was highest in 'Top Green #067' (1.8 µmol/g DW) and lowest in 'Pak Ging' and 'Green Queen' (1.3 µmol/g DW).

ABTS radical scavenging activity of the various broccoli seed extracts

Antioxidant activity of broccoli seed extracts

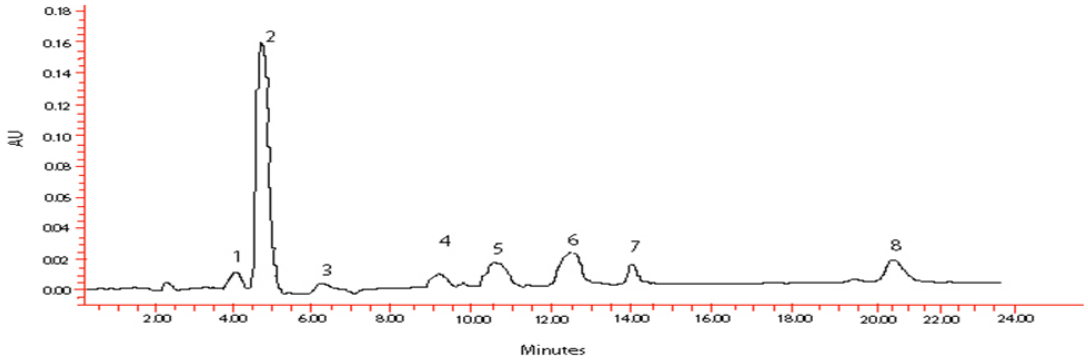

Figure 2. A typical glucosinolate chromatogram from broccoli seeds. Peaks 1: 3-methylsulfinylpropyl-; 2: 4-methylsulfinylbuty-; 3: 5-methylsulfinylpropyl-; 4: 4-hydroxy-3-indolylmethyl-; 5: 4-methylthiobutyl-; 6: 4-methoxy-3-indolymethyl-; 7: 1-methoxy-3-indolylmethyl glucosinolate.

Tabel 1. Total and main individual glucosinolate levels (μmol/g DW) of the five cultivars of broccoli seeds.

Glucosinolates	Cultivars				
	Green King	Packman	Pak Ging	Rod Fai	TopGreen #067
Total glucosinolates	51.2	58.6	25.5	20.3	64.5
4-methylsulphinylbutyl	37.5	44.3	15.8	11.4	48.9
4-hydroxy-3-indolylmethyl	3.1	2.4	2.8	1.6	3.3
4-methylthiobutyl (glucoerucin)	8.9	10.2	5.2	5.6	11.0
4-methoxy-3-indolymethyl (4-Methoxyglucobrassicin)	1.3	1.5	1.3	1.6	1.8
1-methoxy-3-indolylmethyl (neoglucobrassicin)	0.1	0.1	0.2	0.0	0.1

equivalent to that of the three standard antioxidants, i.e. ascorbic acid, quercertin and Trolox, using the ABTS assay, is shown in Figure 3. Results showed that in general the methanolic extract of broccoli seeds gave the highest ABTS radical scavenging activity followed by that of water, ethanol (70%), ethyl acetate and chloroform extracts. The chloroform extracts showed the least scavenging activity (<25%). This activity increased by increasing the concentration of the extracts. The TEAC value of ascorbic acid is highest in 'Pak Ging' (33.2%) followed by 'Packman' (30%), 'Top Green #067' (29.8%), 'Green Queen' (27.2%) and 'Rod Fai' (23%). The TEAC value of quercetin is highest in 'Green Queen' (71.2%) followed by 'Top Green #067' (67.1%), 'Packman' (64.5%), 'Pak Ging' (60.3%) and 'Rod Fai' (53.2%). The TEAC value of Trolox is highest in 'Packman' (39.2%) followed by 'Pak Ging' (38.3%), 'Top Green #067' (36.4%), 'Green Queen' (25.9%) and 'Rod Fai' (19.2%). The highest overall antioxidant activity was in 'Top Green #067' cultivar.

Ferrous ion chelating ability of the broccoli seed extracts

Figure 4 demonstrates the ferrous ion chelating ability of the broccoli seeds extracted with five different solvents. The methanolic seed extract performed the best ferrous ion chelating ability followed by water, ethanol (70%), ethyl acetate and

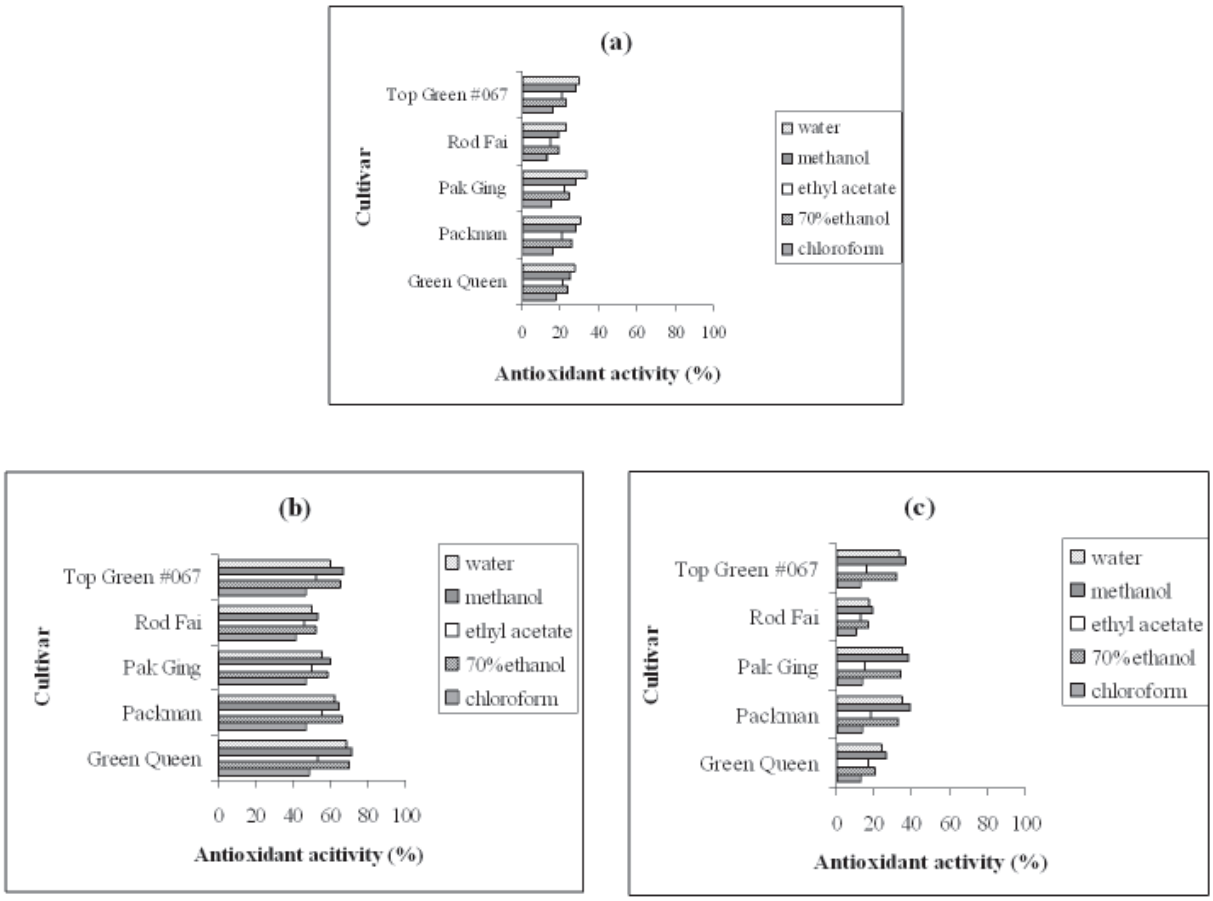

Figure 3. Equivalent antioxidant activity of broccoli seeds extracted with five different solvents as compared to the three standard antioxidants, i.e. ascorbic acid (a), quercetin (b) and Trolox (c), using ABTS assay

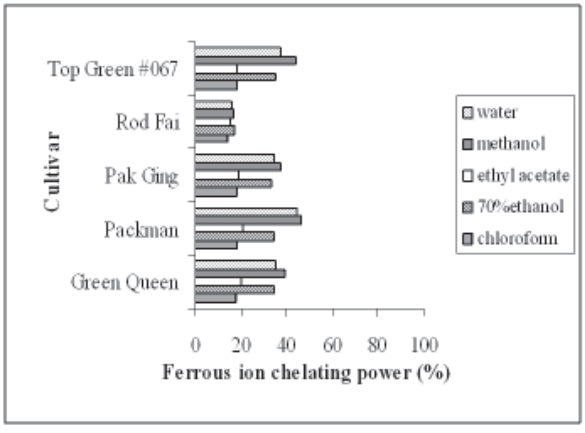

Figure 4. Ferrous ion chelating power of broccoli seeds extracted with five different solvents.

chloroform extracts. 'Packman' showed the highest ferrous ion chelating ability (46.67%), followed by 'Top Green#067' (44.26%), 'Green Queen' (39.21%), 'Pak Ging' (37.65%) and 'Rod Fai' (16.46%).

The results presented in Figures 3 and 4 showed that extraction with methanol and water could give the broccoli seed extracts a higher ABTS radical scavenging activity and ferrous ion chelating ability, though the reason is unclear and needs to be further studied. Ethyl acetate and chloroform extracts were considerably less effective as radical scavenger compared to methanolic extracts. Ethyl acetate extracts were more effective against the ABTS radical than chloroform extracts. As the polarity of these two solvents is quite similar (ethyl acetate is slightly more polar than chloroform) it can be concluded that chloroform is not a suitable solvent for the extraction of radical scavenging compounds from broccoli seeds. Methanol may be the best solvent for such purpose. This result is in accordance with the findings of other researchers (Guo *et al*., 2001; Schlesier *et al*., 2002).

Conclusions

This study has shown that glucosinolates concentrations varied among four Thai broccoli seed cultivars. The highest glucoraphanin concentration was found in 'Top Green #067' cultivar. The antioxidant properties of the four broccoli cultivars, determined by ABTS radical scavenging activity and ferrous ion chelating ability, also showed 'Top Green #067' to be the best. Methanol and water were shown to be the most suitable solvents for the extraction of the antioxidants from broccoli seeds.

Acknowledgments

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