Food and Nutrition Sciences, 2015, 6, 1603-1612
Published Online December 2015 in SciRes. http://www.scirp.org/journal/fns
http://dx.doi.org/10.4236/fns.2015.617165

Low-Dose of the Sulforaphane Precursor Glucoraphanin as a Dietary Supplement Induces Chemoprotective Enzymes in Humans

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Received 26 October 2015; accepted 20 December 2015; published 23 December 2015

Abstract

Broccoli sprout (BS) supplements have been marketed for over a decade for the promising health beneficial effects of sulforaphane (SFN), which induces Nrf2 signaling and downstream chemoprotective genes, including phase 2 enzymes. Most commercially available BS supplements encapsulate heat-processed BS containing glucoraphanin (GR), which is hydrolyzed to SFN by the intestinal microbiota. However, the absorption behavior of SFN following the intake of such BS supplements is still unclear. Additionally, the GR dose (around 30 mg) recommended by many manufacturers of BS supplements is relatively lower than the effective dose determined in previous intervention studies. The aims of this study were to assess the effects of a single administration of a typical BS supplement containing lower doses of GR (30 or 60 mg from 3 or 6 capsules, respectively) on SFN absorption, and also to assess the serum activities of phase 2 enzymes as possible surrogate markers of the beneficial effects of SFN. Urinary excreted isothiocyanates and dithiocarbamates showed that the SFN absorption following administration of BS supplement was prolonged and varied among individuals, which conforms to the well-known characteristics of intestinal microbiota-mediated SFN absorption. The amount of SFN absorbed increased dose-dependently but not linear fashion (9.27 μmol and 13.5 μmol for 3 and 6 capsules, respectively). There was no significant difference in SFN bioavailability and the number of capsules consumed. Serum activities of phase 2 enzymes glutathione S-transferase (GST) and NAD(P)H: quinone oxidoreductase 1 (NQO1), which have been reported to display "chemoprotected states" in organs such as the liver, were dose-dependently and synchronously elevated ($p < 0.05$) following BS supplement intake. This suggests that a low dose of GR (30 mg) exerts chemoprotective effects in humans. In

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How to cite this paper: Ushida, Y., Suganuma, H. and Yanaka, A. (2015) Low-Dose of the Sulforaphane Precursor Glucoraphanin as a Dietary Supplement Induces Chemoprotective Enzymes in Humans. *Food and Nutrition Sciences*, **6**, 1603-1612.
http://dx.doi.org/10.4236/fns.2015.617165

conclusion, our findings will be useful in future clinical studies investigating the chemoprotective effects of SFN, and for the development of BS supplement products.

Keywords

Sulforaphane, Glucoraphanin, Chemoprotection, Broccoli Sprout, Phase 2 Enzymes

1. Introduction

Many epidemiological studies have demonstrated that higher consumption of cruciferous vegetables such as broccoli, kale, and cabbage, is associated with lower risk of various types of cancer and age- and lifestyle-related chronic diseases [1]-[3]. Such beneficial effects have been suggested to be attributable to isothiocyanates (ITCs), which are phytochemicals derived from glucosinolate precursors, and are specifically abundant in cruciferous vegetables [4]. One of the most fascinating ITCs is sulforaphane [SFN; 1-isothiocyanato-4-(methylsulfinyl)-butane], which was identified in broccoli as the most potent naturally occurring inducer of phase 2 enzymes [5]. Within the past two decades, SFN has attracted considerable attention, since it can protect aerobic cells from electrophiles, oxidants, carcinogens, and mutagens by inducing a wide variety of chemoprotective genes, including antioxidant proteins, anti-inflammatory molecules, and phase 2 enzymes by activating Nrf2 (nuclear factor erythroid 2-related factor 2) signaling [6] [7].

SFN has been reported to promote detoxification and elimination of aflatoxin [8], acetaldehyde [9], methylmercury [10], acrolein [11], benzene [12], crotonaldehyde [12] and free radicals [13] through the Nrf2-mediated mechanism. It exerts chemoprotective effects against cancers [14] [15] and chronic diseases such as liver failure [16], type 2 diabetes-induced cardiomyopathy [17], cerebral nerve diseases [18] [19], and macular degeneration [20] in experimental animal models. Additionally, some clinical studies have demonstrated that SFN may be effective in the prevention and/or improvement of skin erythema [21], autism [22], insulin resistance [23], *Helicobacter pylori*-infection [24], and liver abnormality [25].

Broccoli sprouts (BS) are one of the richest dietary sources of glucoraphanin (GR), a glucosinolate precursor of SFN [26]. SFN is readily absorbed from the intestine and excreted into urine as mercapturic acid metabolites within 10 hours of oral intake [27] [28]. However, the stable precursor GR is barely absorbed from the intestine in its intact form; therefore, it needs to be enzymatically hydrolyzed to SFN by myrosinase, a β-thioglucosidase found only in plants and in intestinal microbiota [29]. Previous studies have shown that GR hydrolysis by intestinal microbiota is less efficient than the hydrolysis by endogenous plant myrosinase [27] [28] [30]-[32]. Accordingly, the consumption of cooked or heat-processed BS containing GR without active myrosinase has been reported to result in lower SFN bioavailability compared with fresh BS containing GR and endogenous active myrosinase, or BS preparations, such as smoothies, containing generated SFN [33]-[35]. Although fresh BS and its preparations have demonstrated favorable SFN bioavailability, they have the disadvantage of a short shelf-life due to the instability of the SFN molecule and myrosinase in food matrices. Heat-processed BS products such as hot-water extract and powder have the advantages of longer shelf-life and enriched GR content; hence, they have been used in commercially available BS supplements to provide the health benefits of SFN.

Despite being marketed for more than a decade, there is limited information on the effectiveness of BS supplements. First, the absorption behavior and the bioavailability of SFN after intake of BS supplements in pill and capsule form, remain largely unknown. Second, the clinical efficacy of the dose range of GR that is recommended by commercially available BS supplements has yet to be revealed; the dose range is relatively lower than that shown to be beneficial in previous clinical trials for the prevention and improvement of chronic diseases. To clarify these issues, we assessed the performance of a typical BS supplement product that encapsulates GR-containing powder, on the absorption of SFN. We also assessed its induction potency for serum phase 2 enzyme activities as possible surrogate markers of Nrf2-mediated chemoprotective effects in human subjects.

2. Materials and Methods

2.1. Preparation of BS Supplement Encapsulating GR-Rich BS Extract Powder

BS extract was industrially produced by Kagome Co., Ltd. (Nagoya, Japan). In Brief, BS was harvested 1 day

after germination. BS was then plunged into boiling water and maintained at more than 95˚C for 30 min, and the sprout residues were removed by filtration through a diatomaceous earth. BS extract was concentrated, mixed with dextrin, and then spray-dried. The BS extract powder that is standardized to contain 135 ± 20 mg of GR per gram was blended with waxy cornstarch, crystalline cellulose, calcium stearate, and then encapsulated in hydroxypropyl methylcellulose (HPMC) capsules. A capsule of BS supplement (260 mg in total; contents: 200 mg and HPMC capsule: 60 mg) was designed to contain 10 mg (approx. 22.9 μmol) of GR, which was ascertained by high-performance liquid chromatography (HPLC) analysis with slight modification of Fahey's method [26]. BS supplement was prepared in a good manufacturing practice (GMP) facility (Sansho Pharmaceutical Co., Ltd., Shizuoka, Japan). The nutrition compositions are shown in **Table 1**.

2.2. Study Protocol

The study protocol was approved by the Ethics Committee of Kagome Co. Ltd. (#2009-R04), and was carried out in accordance with the International Ethical Guidelines and Declaration of Helsinki. All subjects gave written informed consent to participate in this study. Twenty-one healthy Japanese male and female volunteers living in Nasushiobara, Tochigi, aged 24 - 60 years, non-smokers, not currently taking medication, and not pregnant were recruited.

The duration of the study was 3 days. Throughout the duration, subjects were asked to avoid consuming alcoholic beverages and foods that are known to contain ITCs and the precursor glucosinolates, such as cruciferous vegetables. Participants received identical meals (total eight meals; three breakfast, three lunch, and two dinner meals). On the morning of day 2, various measurements including body temperature, heart rate, blood pressure, and body weight were noted, and then baseline urine and blood samples were collected, followed by a medical interview. Next, subjects were divided into two groups based on their demographics and characteristics such as gender, age, and body weight. At 11 AM, subjects in the two groups received 3 or 6 capsules of BS supplement containing 30 or 60 mg of GR, respectively. Total urine was collected throughout the study for 30 h (until 5 PM on day 3) as follows; on each urination, subjects were asked to collect urine in plastic bottles, and record the collection time on a designated form. The bottles were stored at approximately 4˚C in cool boxes until collected. Urine volume was measured and the aliquots were frozen at −80˚C until analysis. Whole blood was taken 24 h after administration of BS supplement (11 AM on day 3), and serum samples were prepared and stored at −80˚C until analysis.

2.3. Measurement of Excreted Amount of ITCs and DTCs

Urinary levels of ITCs and their dithiocarbamate metabolites (DTC) were determined by cyclocondensation assay as previously reported [27]. Briefly, each urine sample (5 mL) was centrifuged (300 × *g*, 5 min, 4˚C) to remove particulates. The supernatant (0.5 mL) was transferred into screw-top glass vials containing 1.5 mL of the cyclocondensation reaction mixture (0.5 mL of 500 mM sodium borate buffer (pH 9.25) and 1.0 mL of 1,2-benzenedithiol/methanol). The vials were flushed with nitrogen gas and sealed with screw caps equipped with Teflon-lined septa, and the contents were mixed and incubated for 2 h at 65˚C. The sample was cooled down to room temperature and centrifuged (500 × *g*, 5 min, 4˚C).

Table 1. Nutrition compositions of BS supplement.

	Per 3 capsules[a]
Energy (kcal)	3.0
Protein (g)	0.07
Carbohydrate (g)	0.6
Lipids (g)	0.002
Glucoraphanin, GR (μmol: mg)	68.7 ± 2.3: 30 ± 1
Sulforaphane, SFN (μmol)	<0.5

[a]0.78 g (contents: 0.6 g and capsule: 0.18 g).

The resulted supernatant was injected onto a reverse-phase HPLC column (Partisil 10 μm ODS-2, 4.5 × 250 mm, Whatman, Clifton, NJ) and eluted isocratically with 80% (v/v) methanol/water at a flow rate of 1 mL/min. The cyclocondensation product 1,3-benzodithiole-2-thione, was detected at 365 nm with a photodiode array detector (L-2455, Hitachi, Ltd. Tokyo, Japan). For quantification, a standard curve was constructed using a series of 1,3-benzodithiole-2-thione solutions in 50% (v/v) 2-propanol/water at concentration ranged from 0.01 to 50 μM. The urinary concentrations of ITCs and their DTCs were multiplied by the volume of each urine sample to calculate the amount (μmol) of excreted ITCs and DTCs per urination.

2.4. Measurement of Serum Enzyme Activities of GST and NQO1

Enzyme activities of phase 2 enzymes, glutathione S-transferase (GST), and NAD(P)H: quinone oxidoreductase 1 (NQO1) were measured in serum collected from subjects before and 24 h after administration of BS supplement. The activity of GST was determined with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate as described by Habig *et al.* [36]. NQO1 activities were determined with menadione as substrate by the Prochaska assay, with the exception that the final $NADP^+$ concentration was 200 μM [37] [38].

2.5. Statistical Analysis

Values are shown as mean ± standard deviation. Differences in the values of excreted amount of ITCs and DTCs, and the bioavailability of SFN between the groups were examined by Student's *t*-test. The values of phase 2 enzyme activities before and 24 h after administration of BS supplement were examined by paired *t*-test. Correlation between the percentage changes of enzyme activities of GST and NQO1 was non-parametrically analyzed by the Spearman correlation test. Results were considered statistically significant at $p < 0.05$. For statistical analysis, SPSS for Windows ver. 15.0 (SPSS Japan, Tokyo, Japan) was used.

3. Results

3.1. Subjects Profile

Table 2 shows the subjects profile. There were no significant differences between the two groups in parameters such as gender, age, and body weight. All 21 subjects complied with the study protocol throughout the study period. There was no adverse effect in all the subjects that participated in this study.

3.2. Time Course Changes in Urinary Excretion of ITCs and DTCs

To understand the absorption kinetics of SFN after oral administration of BS supplement at different doses of GR (30 or 60 mg), excreted amounts of ITCs and DTCs were determined in each urine sample. **Figure 1(a)** and **Figure 1(b)** show the excretion curves for individual subjects taking 3 and 6 capsules of BS supplement by cumulating the excreted amounts of ITCs and DTCs determined in each urine samples. Excretions of ITCs and DTCs in the baseline urine samples were at negligible levels in both groups (3 capsules: 0.04 ± 0.03 μmol, 6 capsules: 0.06 ± 0.06 μmol, *N.S.*), which indicated that subjects complied with our request not to have foods containing ITCs and their precursor glucosinolates. The height of the excretion curves largely varied among individual subjects, but the shape of all the curves seemed to be similar; the cumulative amounts of excreted ITCs and DTCs slowly increased in the first few hours, drastically rose up between 8 and 12 hours after dosing GR, and thereafter gradually reached plateau levels. A kinetic analysis showed that Tmax, the time in which maximum excretion of ITCs and DTCs was observed, was equivalent between the low and high GR groups (**Table 3**). When the 30 hour-experimental period was divided into 4 sections, around half of the total excreted DTCs and

Table 2. Subjects profile[a].

Groups	Number	Gender[b]	Age	Body weight (kg)
3 capsules	11	7 (M) 4 (F)	29.1 ± 1.7	60.3 ± 2.8
6 capsules	10	6 (M) 4 (F)	29.6 ± 2.1	60.9 ± 3.2

[a]No significant difference between the groups. [b]M: Male, F: Female.

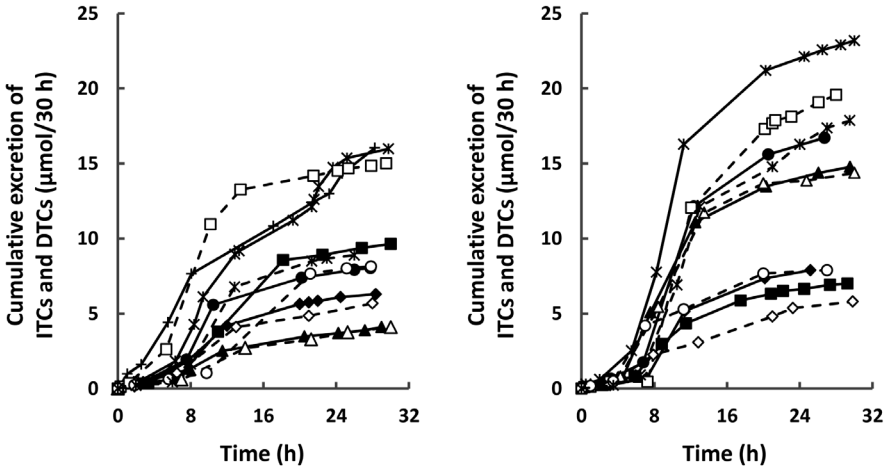


Figure 1. Cumulative amounts of excreted ITCs and DTCs in individual subjects after single administration of 3 and 6 capsules of BS supplement at GR doses of 30 (a) and 60 mg (b) (n = 11 or 10, respectively). Each lines represent data for individual subjects.

Table 3. Urinary Tmax and time-course changes in the excretion of ITCs and DTCs after single administration of BS supplement.

Groups	Tmax (h)[a]	Excreted ITCs and DTCs (μmol)			
		0 - 8 h	8 - 16 h	16 - 24 h	24 - 30 h
3 capsules	12.4 ± 1.3	1.69 ± 0.33	3.84 ± 0.88	2.84 ± 0.70	0.91 ± 0.22
6 capsules	10.7 ± 0.7	1.52 ± 0.37	7.80 ± 1.52	3.20 ± 0.45	0.98 ± 0.19

[a]The time when maximum excretion of ITCs and DTCs was observed. No significant difference between the two groups.

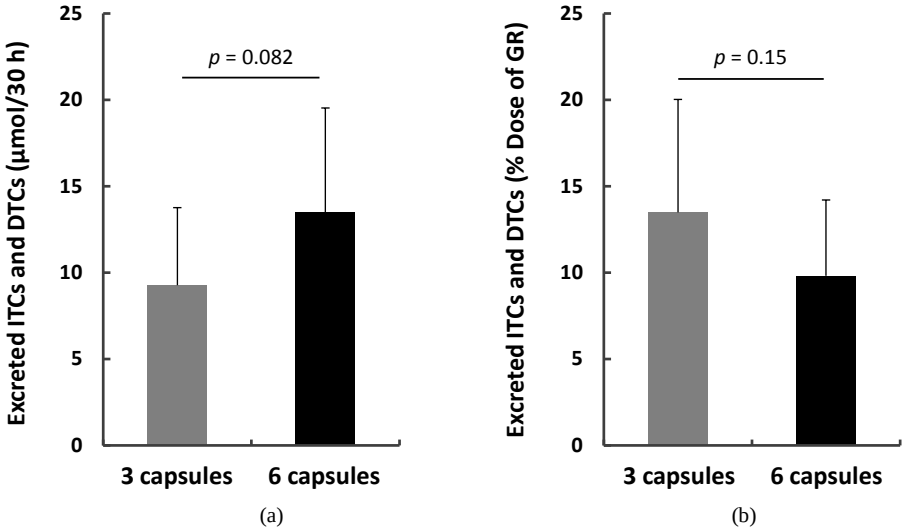
ITCs were observed in urine samples that were collected within 8 to 16 hours. The amount after administration of 6 capsules of BS supplement was two times higher than that after the administration of 3 capsules.

3.3. Comparison of Absorbed Amount of and Bioavailability of SFN between Groups

The amounts of ITCs and DTCs excreted in urine in 30 hours after administration of 3 or 6 capsules of BS supplement were measured to compare the total amounts of absorbed SFN and the bioavailability between the 3 and 6 capsules groups. There was no significant difference in total volumes of urine collected for 30 hours (3 capsules: 1691 ± 818 mL, 6 capsules: 1245 ± 480 mL, *N.S.*). As shown in **Figure 2(a)**, the total amounts of excreted ITCs and DTCs (indicator of absorbed amount of SFN), appears to be dose-dependent. The ratios of the total amounts of urinary ITCs and DTCs (μmol) to the doses of GR (68.7 or 137.4 μmol, for 30 or 60 mg of GR, respectively) were calculated as the bioavailability of SFN for individual subjects. **Figure 1(a)** and **Figure 1(b)** show the bioavailability of SFN largely varied among the individuals (5.1% - 23.3%). No significant difference was observed in the bioavailability of SFN between the two groups, but the mean value was apparently higher in the 3 capsules group (13.5%) than the 6 capsules group (9.8%) (**Figure 2(b)**).

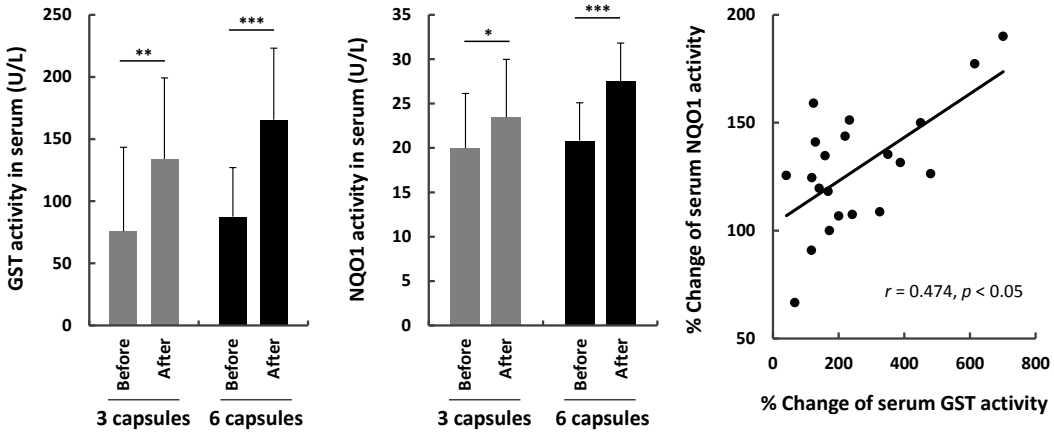
3.4. Serum Enzyme Activities of GST and NQO1

To assess whether single administration of BS supplement, at lower dose of GR (30 - 60 mg) than previous studies, induces phase 2 enzymes that are present in the downstream of the Nrf2 signaling, enzyme activities of GST and NQO1 were measured in serum samples obtained from the subjects before and 24 h after the administration. As shown in **Figure 3(a)** and **Figure 3(b)**, single administration of 3 or 6 capsules of BS supplement at GR doses of 30 or 60 mg significantly increased serum enzyme activities of both GST and NQO1. Dose-dependent inducer effects were observed for both enzymatic activities, GST and NQO1 activities were increased



(a) (b)

Figure 2. Total amounts of ITCs and DTCs excreted during 30 h after single administration of 3 and 6 capsules of BS supplement (n = 11 and 10, respectively) (a) and the percent ratio to GR doses (b). *p* values were analyzed by student's *t*-test.



(a) (b) (c)

Figure 3. Serum activities of phase 2 enzymes, GST (A) and NQO1 (B) in subjects before and 24 after single administration of 3 and 6 capsules of BS supplement (n = 11 and 10, respectively), and the relationship between the percentage changes of GST and NQO1 activities in individual subjects (C). *: $p < 0.05$, and ***: $p < 0.001$ (paired *t*-test). Correlation coefficient (*r*) and significance (*p*) were analyzed by the Spearman correlation test.

by 1.7- and 1.2-fold in the 3 capsules group and by 1.9- and 1.3-fold in the 6 capsules group, respectively. Percentage changes in individual subjects' serum activities of GST and NQO1 were plotted in **Figure 3(c)**. Spearman correlation test revealed that there was a significant positive relationship between the changes in the activities of GST and NQO1, which suggested that SFN might activate Nrf2 signaling *in vivo*.

4. Discussion

Several studies have shown that SFN can be absorbed into the body due to the intestinal microbiota possessing GR hydrolysis activity, even when cooked or heat-processed BS containing GR without active myrosinase is consumed [27]-[34]. However, it has been largely unclear whether SFN is absorbed after taking BS supplements in which heat-processed BS is encapsulated. The present study revealed that the absorption of SFN occurred after the administration of BS supplement, which was confirmed by measuring the excreted amounts of ITCs and DTCs in urine samples, consistent with many previous studies [27] [29] [30]. The bioavailability (5% - 23%) of

SFN observed in the present study is within the range of those in previous studies (2% - 40%), where cooked and heat-processed BS, not in capsule form, had been used. The only concern is that the bioavailability of SFN seemed to be lower after taking 6 capsules of BS supplement (60 mg of GR) compared to that after taking 3 capsules (30 mg of GR), which suggested the possibility of overcapacity of the microbiota-derived myrosinase activity to higher dose of GR. However, the excreted amount of ITCs and DTCs in the time range of 8 to 16 h (around Tmax) increased directly proportional to the GR dose (**Table 2**), indicating that myrosinase-mediated conversion from GR to SFN was unlikely to be limited within the dose range of GR (up to 60 mg = approximately 137 μmol) investigated in the present study. This was supported by previous studies [29], in which the bioavailability of SFN after GR dose of 200 μmol (45 Baltimorean; mean 11.8%, median 9.6%) was quite similar to that after GR dose of 400 μmol (99 Chinese; mean 10.4%, median 9.4%). We consider the encapsulation of BS powder has little influence on the absorption and bioavailability of SFN.

Over the past decade, chemoprotective effects of SFN in human subjects have been demonstrated in some clinical trials, most of which have used BS as a dietary source of SFN. The dose of GR (or sometimes SFN) varied depending on how to give BS to human subjects. In previous studies using fresh BS preparations containing generated SFN or GR with active myrosinase, the daily doses were often around 100 μmol [22] [23]. While in studies that used heat-processed BS containing GR but without active myrosinase, the daily doses of GR were at relatively higher levels, in consideration of the lower bioavailability of SFN. For instance, in several studies carried out in Qidong, China, which demonstrated the chemoprotective effects of SFN against carcinogens and pollutants such as aflatoxin, benzene, and acrolein, the daily GR dose was within 400 to 800 μmol [8] [11]. These clinical trials reported no adverse events caused by GR intake, indicating GR is likely to be safe for human. Nonetheless, if being provided as dietary supplements, the GR dose should be considered carefully, because dietary supplements are consumed by a wide variety of people with few restrictions, and pose potential health risks by overdose and improper usage. Previous surveys have reported that the estimated daily intake of glucosinolates is around 100 μmol [39] [40]; this dose has been regarded as a safe daily dose of GR for a diverse group.

In the present study, we showed that single administration of BS supplement at GR dose of 30 - 60 mg (approximately 69 - 138 μmol) synchronously increased serum activities of phase 2 enzymes such as NQO1 and GST in human subjects. A previous study using a mouse model reported that activities of these phase 2 enzymes in serum were induced in conjunction with those in the liver, by the classical inducer butylated hydroxyanisole (BHA), and would be a possible surrogate marker of the “chemoprotected state” of tissues [41]. In human studies, serum phase 2 enzymes including GST have been used as markers for preliminary evaluation of potential chemoprotective effects of vegetables and phytochemicals [42]-[44]. The present study suggested the possibility that the intake of BS supplements at 30 - 60 mg of GR showed chemoprotective effects in human subjects, through inducing phase 2 enzymes (also Nrf2 signaling) in various tissues. Therefore, this study could provide useful information for future clinical trials and further development of BS supplements containing GR. To our knowledge, such a low dose of GR has not been evaluated in clinical trials in the past, except in a very recent study [25]. In order to demonstrate the chemoprotective effects of BS supplements at low doses of GR, randomized studies are being planned or are in progress at our institute.

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