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Simultaneous Hydrolysis and Extraction Increased Erucin Yield from Broccoli Seeds

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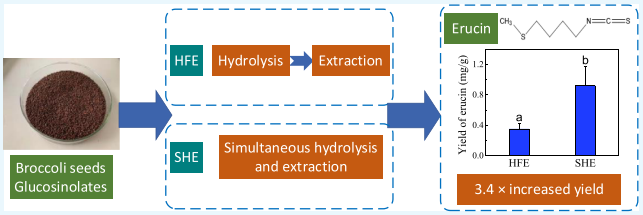
ABSTRACT: Isothiocyanates (ITCs) are well-known chemopreventive agents that have received significant interest across the nutrition and pharmaceutical industries owing to their anticancer properties, thus it is essential to increase the conversion of glucosinolate to ITCs by myrosinase to maximize their health benefits. In this paper, using broccoli seed meals as a raw material, we comparatively analyzed the outcomes of two extraction methods: (i) hydrolysis followed by extraction (HFE) and (ii) simultaneous hydrolysis and extraction (SHE) in terms of the ITC yield. The results revealed that the SHE method showed a relatively greater erucin production from broccoli seeds and greater antitumor and antioxidant activities. A similar phenomenon was found for the hydrolysates of crude myrosinase and crude glucosinolate separated from broccoli seeds. However, when the crude glucosinolates were hydrolyzed by purified broccoli myrosinase, or when pure glucoraphanin was hydrolyzed by crude myrosinase, no significant effects were noted on the types and yields of ITCs between the SHE and HFE methods.



INTRODUCTION

In recent years, the consumption of broccoli has been increasingly advocated for effectively reducing the risk of some chronic diseases, such as obesity, type 2 diabetes, cardiovascular diseases, cancer, and osteoporosis.[1−5] The beneficial properties of broccoli can be mainly attributed to its bioactive phytochemicals, including hydroxycinnamic acids, flavonoids (e.g., quercetin, kaempferol and myricetin), carotenoids (lutein), and glucosinolates, in which glucosinolates are of the most interest.[6]

During chewing or cutting, broccoli cells are damaged, the glucosinolates are released and converted by endogenous myrosinase to produce D-glucose and thiohydroximate-*O*-sulfinate, the latter is unstable and immediately rearranged into sulfate and isothiocyanate (ITC), nitrile or thiocyanate, depending on the pH, epithiospecifier protein (ESP), ascorbic acid, Fe^{2+}, and so forth.[7] Glucoraphanin is the main glucosinolate present in broccoli, accounting for approximately 80% of the total yield, along with the presence of other glucosinolates (such as glucobrassicin, 4-methoxyglucobrassicin, and 1-methoxyglucobrassicin) in smaller amounts.[8] The hydrolysis of glucoraphanin by myrosinase forms sulforaphane (4-(methylsulfinyl) butyl isothiocyanate, SF). Erucin (4-(methylthio) butyl isothiocyanate, ER) as the reduced analogue of SF is the product hydrolyzed by glucoerucin, which is also present in broccoli, especially in broccoli sprouts (Figure S1).[9,10]

ER and SF are similar in structure because they both possess an aliphatic side chain that supports a similar pharmacokinetic outcome. The protective effects of SF and ER against prostate, pancreas, lung, liver, and colon cancer in several tissues and cancer cells have been proved by several past studies.[11−13] One major mechanism of action of SF is the upregulation of several phase II detoxification enzymes, including NAD(P)H:quinone oxidoreductase 1 (NQO1), which provides protection against oxidative stress and exhibits versatility by acting as a cytoprotective enzyme.[14] In most cases, SF and ER exert similar effects.[10]

As structurally related compounds, glucoraphanin and glucoerucin or SF and ER can be transformed into each other both *in vitro* and *in vivo*. The *in vivo* studies on humans and rats have reported the reduction of SF to ER, and the conversion of glucoraphanin/SF to reduced glucoerucin/ER occurred in the liver, with hydrolysis and enterohepatic circulation of glucosinolates caused by the gut microbes.[15,16] After the mice were fed with purified SF or broccoli sprout powders, ER was the favored form in the liver, kidney, and bladder, even when only pure SF was consumed.[17] In an *in vitro* study, glucoerucin was oxidized to glucoraphanin by hydrogen peroxide,[18] and ER was oxidized to SF under transition-metal-free conditions (hydrogen peroxide/glacial acetic acid).[19]

Alyssin (5-methylsulfinylpentyl isothiocyanate), iberin (methylsulfinylpropyl isothiocyanate), and iberverin (1-iso-

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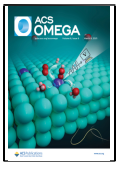

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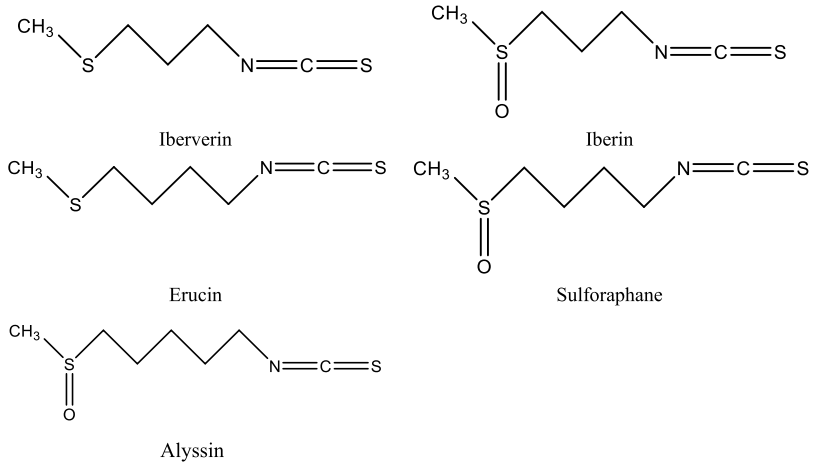

Figure 1. Structures of iberverin, iberin, erucin, sulforaphane, and alyssin.

Table 1. Effects of HFE and SHE on the Changes in Types and Content of ITCs from Broccoli Seeds[a]

	cultivars					
	LS-1		LS-2		Xinfeng	
ITCs	HFE	SHE	HFE	SHE	HFE	SHE
3-BITC	0.15 ± 0.01[a]	0.17 ± 0.01[a]	-[a]	-[a]	-[a]	-[a]
iberverin	0.14 ± 0.00[a]	0.17 ± 0.01[b]	0.13 ± 0.01[a]	0.14 ± 0.00[a]	0.15 ± 0.00[a]	0.26 ± 0.01[b]
ER	0.35 ± 0.07[a]	0.92 ± 0.25[b]	0.41 ± 0.06[a]	0.80 ± 0.24[a]	0.32 ± 0.05[a]	1.08 ± 0.07[b]
PEITC	0.14 ± 0.00[a]	0.14 ± 0.00[a]	0.14 ± 0.00[a]	0.14 ± 0.01[a]	0.17 ± 0.02[a]	0.18 ± 0.00[a]
iberin	0.27 ± 0.04[a]	0.25 ± 0.03[a]	0.16 ± 0.03[a]	0.13 ± 0.01[a]	0.37 ± 0.12[a]	0.33 ± 0.01[a]
SF	1.30 ± 0.35[a]	1.34 ± 0.35[a]	2.11 ± 0.29[a]	1.63 ± 0.41[a]	0.85 ± 0.34[a]	0.70 ± 0.03[a]
alyssin	0.13 ± 0.00[a]	0.13 ± 0.01[a]	0.14 ± 0.01[a]	0.13 ± 0.00[a]	-[a]	-[a]
ERN	0.16 ± 0.01[a]	0.16 ± 0.02[a]	0.26 ± 0.02[a]	0.22 ± 0.04[a]	0.14 ± 0.00[a]	0.13 ± 0.00[a]
SFN	0.15 ± 0.00[a]	0.13 ± 0.00[a]	0.17 ± 0.02[a]	0.17 ± 0.02[a]	-[a]	-[a]
total ITCs*	30.63 ± 2.78[a]	34.51 ± 2.32[a]	44.26 ± 5.98[a]	35.33 ± 11.38[a]	18.52 ± 1.41[a]	25.90 ± 0.85[b]

[a]The unit of each ITC is mg/g, while the unit of total ITCs is μmol/g. The data are represented as mean ± SD (n = 3). [a,b]Values in the same seed column with different superscripts are significantly different (P < 0.05). "-": not detected. 3-BITC: 3-butenyl isothiocyanate; ER: erucin; PEITC: phenethyl isothiocyanate; SF: sulforaphane; ERN: erucin nitrile; and SFN: sulforaphane nitrile. *Types and contents of ITCs were measured by GC−MS (mg/g), while total ITCs were measured by the cyclocondensation method through HPLC (μmol/g).

thiocyanato-3-(methylthio) propane) are also analogues of SF (Figure 1). Considering their structures, alyssin differs from SF only in having an extension of its methylene chain by one $-CH_2-$ group, while iberin is shortened by one $-CH_2-$ group, and iberverin is the reduced analogue of iberin. Iberin, SF, and alyssin increase the intracellular reactive oxygen species and inhibit tubulin polymerization in HepG2 cells, which result in the cell cycle arrest in the S and G_2/M phase, among which, alyssin possesses the highest antitumor activity.[20] SF, iberin, and iberverin from oxheart cabbage seed extract can inhibit the growth of A549 cells *in vitro* and exhibit inhibitory activity on A549 cells in the following order: SF > iberin > iberverin.[21]

The common method of SF preparation from broccoli include an enzymatic conversion process, followed by an extraction process.[22−24] Generally, there are two hydrolysis and extraction methods, namely, hydrolysis followed by extraction (HFE) and simultaneous hydrolysis and extraction (SHE), of which the HFE method is more commonly applied. In our previous study, we have reported that ethyl acetate and potassium phosphate buffer were added simultaneously to the seed meal and agitated for some period of time,[25] but the effects of the hydrolysis methods on SF and ITCs yields have not yet been compared. In this paper, the ITC yields including SF and ER extracted by HFE and SHE methods from broccoli seeds were comparatively analyzed. Moreover, the enzymatic hydrolysis of ITCs from glucosinolate was affected by several factors, such as the temperature, pH, and Fe^{2+}, which have all been well studied. However, to the best of our knowledge, the effects of organic solvents on the myrosinase and ITC yields are not yet reported. Our pre-experiment study revealed that, in Brassica vegetable seed meal, organic solvents such as ethyl acetate and dichloromethane exert significant effects on the production of ITCs (data not shown). In this paper, the effects of hydrolysis methods on the yields of SF and ER were studied and the possible mechanisms involved have been discussed.

RESULTS

Effects of Hydrolysis and Extraction Methods on the Hydrolysates of Broccoli Seeds. It is essential to optimize the conversion of glucosinolates to ITCs by myrosinase to maximize its health benefits. In this study, the effects of HFE and SHE methods on the yields of ITCs from the 3 different cultivars of broccoli seeds were analyzed (Table 1), which indicated that the extraction methods significantly affected the yield of total ITCs from broccoli seeds (P < 0.05). The total ITCs from broccoli seeds extracted by SHE varied with the different cultivars, in which ITC yield of Xingfeng was

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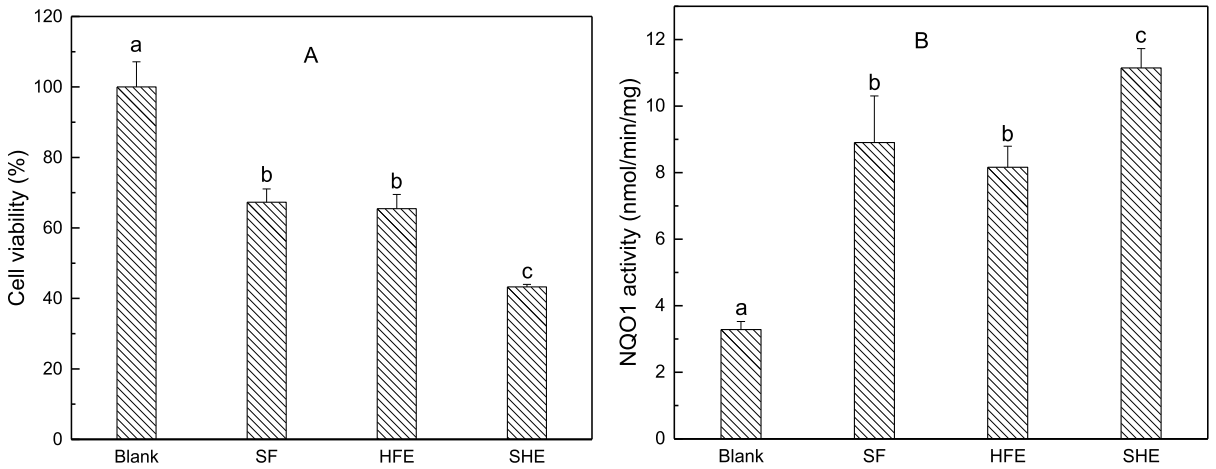

Figure 2. Effects of the broccoli seed extract through HFE and SHE methods on the viabilities (A) and NQO1 activities (B) of the B16 cell. The data are represented as mean ± SD (n = 3). [a−c]Values with different superscripts are significantly different (P < 0.05).

Table 2. Effects of HFE and SHE on the Types and Contents of TICs Generated by Crude Broccoli Myrosinase and Crude Glucosinolates, the Unit of Each ITC is mg/G, While the Unit of Total ITCs is μmol/G[a]

methods	3-BITC	iberverin	ER	iberin	SF	alyssin	SFN	total ITCs*
HFE	0.16 ± 0.06[a]	-[a]	0.17 ± 0.06[a]	0.18 ± 0.05[a]	3.47 ± 1.05[a]	0.08 ± 0.02[a]	0.07 ± 0.00[a]	41.36 ± 10.27[a]
SHE	0.25 ± 0.04[a]	0.08 ± 0.00[b]	1.91 ± 0.23[b]	0.17 ± 0.02[a]	4.88 ± 0.80[a]	0.10 ± 0.01[a]	0.10 ± 0.01[b]	70.16 ± 3.46[b]

[a]The data are represented as mean ± SD (n = 3). [a,b]Values in the same column with different superscripts are significantly different (P < 0.05). *Types and contents of ITCs were measured by GC−MS (mg/g), while total ITCs were measured by the cyclocondensation method through HPLC (μmol/g).

Table 3. Effects of HFE and SHE on the Kinds and Contents of ITCs Generated by Purified Broccoli Myrosinase and Crude Glucosinolates, the Unit of Each ITC is mg/G, While the Unit of Total ITCs is μmol/G[a]

methods	3-BITC	iberverin	ER	SF	total ITCs*
HFE	0.12 ± 0.08[a]	-[a]	1.44 ± 0.05[a]	2.62 ± 0.16[a]	54.40 ± 0.27[a]
SHE	0.14 ± 0.06[a]	0.05 ± 0.00[b]	1.66 ± 0.38[a]	3.03 ± 1.17[a]	60.55 ± 4.44[a]

[a]The data are represented as mean ± SD (n = 3). [a,b]Values in the same column with different superscripts are significantly different (P < 0.05). *Types and contents of ITCs were measured by GC−MS (mg/g), while total ITCs were measured by the cyclocondensation method through HPLC (μmol/g).

increased by 1.40-folds (P < 0.05), while LS-2 was decreased slightly. We analyzed totally 6 broccoli cultivars (data not shown), with LS-2 as the only exception.

The effects of HFE and SHE on the changes in the types and yields of ITCs from broccoli seeds were analyzed by gas chromatography−mass spectrometry (GC−MS) (Table 1). When compared with the HFE group, the most significant change in the SHE group is ER (P < 0.05), which was increased by 2.63- to 3.38- folds. Another significant change in the yield was noted for iberverin in the LS-1 and Xingfeng seeds but not in the LS-2 seeds. The yields of other ITCs containing sulfoxide groups, such as alyssin, SF, and iberin, did not change significantly. In addition to ITCs, the nitriles including erucin nitrile (ERN) and sulforaphane nitrile (SFN) were also detected in the hydrolysates, although the yields of the two nitriles in the 3 broccoli cultivars showed no significant differences (P > 0.05).

The cell inhibition and NQO1 inducing activities of LS-1 broccoli seed hydrolysates extracted by the SHE and HFE methods were analyzed. The extracts significantly inhibited the viability of B16 cells (P < 0.05), and extraction through the SHE method inhibited the B16 cells to a greater degree than that through the HFE method (Figure 2A). The NQO1 inducing analysis revealed that the SHE method increased the NQO1 activity of the extracts by 37% when compared to that by the HFE method (Figure 2B).

Effects of Hydrolysis and Extraction Methods on the Hydrolysates of Crude Myrosinase and Crude Glucosinolates. Crude glucosinolates and crude myrosinase were extracted from broccoli, respectively, and the total ITCs generated from crude glucosinolates hydrolyzed by crude broccoli myrosinase were analyzed (Table 2). The SHE methods significantly increased the yield of total ITCs by 1.7-fold (P < 0.05). To detect the differences in the types and yields of ITCs, the hydrolysates were analyzed by GC−MS. As shown in Table 2, when the crude broccoli myrosinase hydrolyzed crude glucosinolates through the SHE method, the yield of ER and SF increased to 1.91 and 4.88 mg/g, which were 11.4-fold and 1.4-fold higher than that through the HFE method, respectively.

Effects of Hydrolysis and Extraction Methods on the Hydrolysates of Purified Myrosinase and Crude Glucosinolates. To analyze the effects of different extraction methods (SHE and HFE) on ITCs produced from seeds, the myrosinase was purified from broccoli seeds via gel filtration. The crude broccoli myrosinase extracts were separated into 4 peaks through the Sephadex G-75 (Figure S2). As indicated in Figure S3, using sinigrin as a substrate, the ability of peak 1 to

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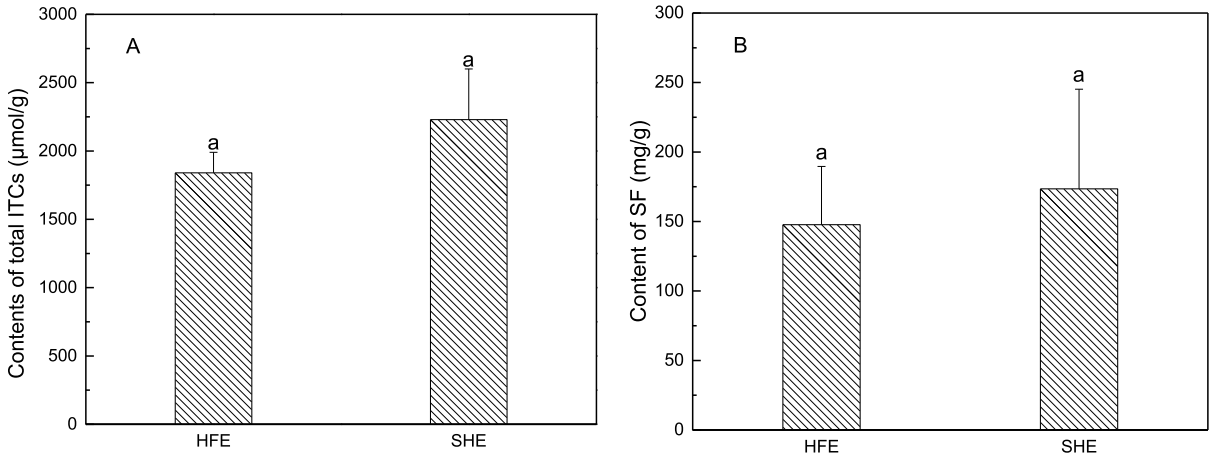

Figure 3. Effects of HFE and SHE methods on the yields of total ITCs (A) and SF (B) generated by crude broccoli myrosinase and pure glucoraphanin. The data are represented as mean ± SD (n = 3). Same letter indicates insignificant difference (P > 0.05).

produce ITC was 63-fold higher than that of the crude enzyme. The activities of crude myrosinase in peaks 2 to 4 were also much weaker than that in peak 1.

The broccoli myrosinase from peak 1 provided a strong protein band on the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel corresponding to 32 kDa (Figure S4). The broccoli myrosinase from peaks 2 to 4 showed the same soluble fractions. However, their grayscale was weaker than that of broccoli myrosinase from peak 1. The crude extract contained only a few other proteins (25−32 kDa), which may demonstrate the myrosinase activity and hydrolyze glucosinolates to proudce ITCs.

The total ITC yield obtained from crude glucosinolates hydrolyzed by purified broccoli myrosinase were analyzed (Table 3). Unlike hydrolysis with crude myrosinase, the effect of the SHE method on crude glucosinolates hydrolyzed with purified broccoli myrosinase was insignificant and similar to that by the HFE method (P > 0.05). The crude broccoli myrosinase hydrolyzed the crude glucosinolates to generate ITCs in a slightly greater yield than that with purified broccoli myrosinase through the SHE method.

The crude glucosinolates hydrolyzed with purified broccoli myrosinase were analyzed by GC−MS (Table 3), in which the SHE and HFE methods had the same effect on the types and yields of ITCs (except for iberverin). Notably, the yield of ER and SF was not affected by the selected hydrolysis and extraction methods (P > 0.05). The types of ITCs generated from crude glucosinolates hydrolyzed by purified broccoli myrosinase were much fewer than those by crude broccoli myrosinase through both the SHE and HFE methods. For example, iberin and alyssin were not detected in the hydrolysate of purified myrosinase. In addition, no nitrile was detected in the ITCs generated with purified broccoli myrosinase, indicating that ESP was removed via gel filtration.

Effects of Hydrolysis and Extraction Methods on the Hydrolysates of Crude Myrosinase and Pure Glucoraphanin. The total yield of ITCs and SF generated with crude myrosinase and pure glucoraphanin through the HFE and SHE methods were analyzed by reverse phase high-performance liquid chromatography (RP-HPLC) and GC−MS, respectively (Figure 3). As indicated in Figure 3A, the difference in the yields of total ITCs in the hydrolysates of crude myrosinase and glucoraphanin through the HFE and SHE methods was insignificant (P > 0.05). In addition, only the SF was detected, which indicated that the hydrolysis of glucoraphanin could not produce ER (Figure 3B), and the yield of SF was not affected by the selected method (P > 0.05).

DISCUSSION

Broccoli seeds can produce the highest amounts of SF (approximately 4.47- to 13.19-folds than that of broccoli spouts) and hence are a suitable raw material for preparing SF.[24] Liang et al.[31] reported that broccoli seeds produced up to 4.75 mg/g SF. However, Lv et al.[23] reported that SF production in various broccoli seeds ranged from 2.43 to 12.07 mg/g, much higher than that reported by Liang et al.[31] These differences may be attributed to the difference in the analytical methods, and SF yield varied significantly among the different cultivars.[23] In this study, the ITC yield ranged from 1.84 to 4.41 mg/g, while the SF yield ranged from 0.70 to 2.11 mg/g, these results were consistent with those reported by Liang et al.[32]

The precursor of iberin, ER, SF, and alyssin have been reported to be present in several *Brassica* plants. For example, glucoalyssin, a precursor of alyssin, was detected in *Alyssum* (*Brassicaceae*) plants, which contains a high amount of total glucosinolates ranging from 9.90 to 135.40 μmol/g dried material and can be used as an alternative source of total ITCs and alyssin.[20] ER has been identified as a major component derived from Arugula and Siberian wallflower seed meal, and glucoiberin is abundantly present in Lesquerella seed meal, the average yield of glucoiberin is 65.00 ± 0.80 mg/g of defatted seed meal, while the molar yield of iberin is 48.6% from glucoiberin.[33] In this study, although SF was the main hydrolysate obtained from broccoli seeds, the analogues of SF (such as ER, alyssin, iberin, and iberverin) were also detected in the hydrolysates (Table 1). Interestingly, the productions of ER and iberverin but not SF and iberin were affected by the addition of the organic solvent, that is, the SHE method had higher ER and iberverin yields, but there was no significant difference in the iberin, SF, and alyssin yields between the two methods (Table 1). Moreover, because ER and iberverin have similar activities with SF and iberin,[10] the SHE method increased the cell inhibition and NQO1-inducing activities of hydrolysate (Figure 2). Therefore, the SHE method was more suitable for the extraction of ITCs from broccoli seeds.

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In the Introduction section, we have discussed that glucoraphanin and glucoerucin or SF and ER can be transformed into each other both *in vitro* and *in vivo*. However, these mechanisms do not explain how the production of ER was increased in the present work. The ER concentration was much higher when the broccoli seed meal was extracted by the SHE method than by the HFE method (Table 1). A similar phenomenon was recorded for the hydrolysates of crude myrosinase and crude glucosinolate (Table 2). However, when myrosinase was purified by gel filtration, no significant difference was noted in the ITC yields between the HFE and SHE methods (Table 3), and when pure glucoraphanin was hydrolyzed with crude myrosinase, ER was not detected (Figure 3). Based on these experiments, we deduced that although organic solvents showed no effect on the myrosinase activity, some proteins other than myrosinase may have produced ER in the action of the organic solvent.

Beside myrosinase, ESP, thiocyanate forming proteins (TFP), and nitrile-specifier proteins (NSP) in Brassica vegetables may affect the yield of ITCs. ESP and NSP could shift the hydrolysis of glucosinolate in favor of ITC nitrile instead of ITCs within the Lossen-type rearrangement,[34] while TFP is termed so owing to its ability to form thiocyanate.[35] The temperature, pH and Fe^{2+} may affect the stability and activity of ESP, TFP, and so forth, and thus decreased the yield of ITCs. In this experiment, the yield of SFN in two broccoli groups showed no significant diffidence (Table 1), implying that ethyl acetate has no effect on the activity of ESP. However, whether these proteins with myrosinase produce ER and whether any protein plays an important role in the conversion of sulfoxide SF to thioether ER remain to be evaluated in further studies.

CONCLUDING REMARKS

In the present study, the effects of HFE and SHE methods on the yields of ITCs from broccoli seeds were analyzed, the SHE method showed a relatively greater ER production from broccoli seeds and greater antitumor and antioxidant activities. Interestingly, the productions of ER and iberverin but not SF and iberin were affected by the addition of the organic solvent. A similar phenomenon was found for the hydrolysates of crude myrosinase and crude glucosinolate. However, no significant difference was recorded in the ITC yields between the HFE and SHE methods when pure myrosinase or pure glucoraphanin were employed. Some proteins other than myrosinase may have produced ER in the action of the organic solvent.

EXPERIMENTAL PROCEDURE

Materials. In this work, three cultivars of broccoli seeds were employed, in which LS-1 and LS-2 were preserved at Zhejiang Provincial Key Lab for Chem & Bio Processing Technology of Farm Products, and Xingfeng was provided by Qingfengyingke Seed Co., Ltd. (Guangdong, China). The moisture contents of LS-1, LS-2 and Xingfeng were 7.8 ± 0.3, 8.0 ± 0.2, and 9.3 ± 0.3%, respectively. Column chromatography materials were purchased from GE Healthcare (Pittsburgh, USA). Myrosinase, sinigrin, SF, and the reagents of NAD(P)H:quinone oxidoreductase 1 (NQO1) determination were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Glucoraphanin with 98% purity was purchased from J&K Chemical Ltd. (Shanghai, China), and iberin, iberverin, phenethyl isothiocyanate (PEITC), 3-BITC, and alyssin for GC calibration were also purchased from J&K Chemical Ltd. (Shanghai, China). The B16 cells was purchased from Nuoyang Biotechnology Co., Ltd. (Hangzhou, China). The materials of cell culture were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The other chemicals used in the study were purchased from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China).

Extraction of ITCs. To analyze the differences in the content of total ITCs from broccoli seeds and different extraction methods, broccoli seeds were crushed by a grinder (DXF-20D, Daxiang, China) to particle sizes of 0.5−1 mm. Crushed seed meals (30 g) was added to petroleum ether (210 mL) and shaken for 2 h, then the petroleum ether layer was removed by filtration. The defatted seed meal was dried in a fume hood overnight at room temperature. The dried defatted seed meal was divided into two groups. The first group was HFE, in which the defatted seed meal was added with 15 mL of the phosphate buffer (0.1 M, pH 7.0) and was shook for 2 h, after which 35 mL of ethyl acetate was added to the mixture and shaken for 4 h, then 2 g of sodium chloride was added to the solution. After shaking for another 15 min, the ethyl acetate layer was separated and the residue was washed with ethyl acetate for another two times. The ethyl acetate layer was combined and added with anhydrous sodium sulfate (10 g) to remove water. The combined ethyl acetate was evaporated by a Rotavapor at a temperature of 60−70 °C, the residue was dissolved in 5 mL of methanol and stored at −20 °C. Another group is SHE, which was similar with the HFE group, except that the defatted seed meal was added with 15 mL of phosphate buffer (0.1 M, pH 7.0) and 35 mL of ethyl acetate at the same time and was shook for 4 h.

Extraction of Crude Glucosinolates. The crude glucosinolates were extracted according to the method of Sarvan et al.[26] with some modifications. The broccoli seeds (30 g) were kept at 100 °C for 5 h to inactivate the myrosinase, and the seeds were defatted. The defatted seed meal was added to 70 mL of 70% methanol with glass beads and the glucosinolate was extracted by a Soxhlet extractor. The crude glucosinolate extract was concentrated and maintained at −80 °C for the subsequent experiments.

Extraction and Purification of Broccoli Myrosinase. The crushed broccoli seeds were added to phosphate buffer [pH 6.8, 1 mM dithiothreitol, 3 mM ethylenediaminetetraacetic acid (EDTA), and 5% glycerin] and disrupted by sonication for 20 min at 4 °C, after which the crude extract was centrifuged at 6000*g* for 40 min. Ammonium sulfate was added to the supernatant to 55% saturation and kept for 2 h at 4 °C. The mixture was centrifuged at 6000*g* for 40 min (4 °C), then the precipitate was dissolved in phosphate and centrifuged at 7000*g* for 10 min. The clear supernatant containing crude myrosinase was collected and dialyzed through a dialysis bag (8−14 kDa). Thereafter, the crude enzyme solution was lyophilized to powder for further experiments.

For further purification, the crude myrosinase powder was well dissolved in 10 mM phosphate buffer (pH 6.5). The above crude myrosinase obtained was passed through a column (D 2 × 70 cm) of Sephadex G-75 that was pre-equilibrated with 10 mM phosphate buffer (pH 6.5). Elution was achieved with 10 mM phosphate buffer at a flow rate of 0.3 mL/min. The purified fractions with myrosinase activity were collected, dialyzed, and lyophilized for further research.

Purity of Myrosinase and Protein Determination. The purity and molecular weight of myrosinase were analyzed by

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SDS-PAGE on a 12% resolving slab gel system according to the method of Zhang et al.[27] The protein concentrations were determined by the Bradford method.[28]

B16 Murine Melanoma Cell Viability Analysis. The B16 murine melanoma cells were cultured according to the method of Qarawi et al.[29] The cells were cultured in RPMI 1640 media (Gibco) containing heat-inactivated foetal bovine serum (10%, ICN), L-glutamine (2 mM), nonessential amino acids (1%), penicillin (50 U/mL), and streptomycin (50 μg/mL) in a humidified atmosphere of 95% of air and 5% of CO_2.

The B16 murine melanoma cell viability was monitored by the tetrazolium dye colorimetric test according to the method of Chiang et al.[30] with some modifications. Briefly, the cells in the logarithmic growth phase were digested with trypsin containing EDTA to prepare a single cell suspension, after which the cells were inoculated in 96-well microplates (2×10^4 cells/well in 100 μL of medium) and cultured with 5% of CO_2 at 37 °C for 24 h. Cells were then washed with phosphate-buffered saline (PBS) and incubated with the SF solution (30 μM) and the extracts (30 μM) from the broccoli seed meal through the HFE method for 24 h, respectively. The extracts through the SHE method were diluted by the same multiple with the HFE method. The solution without SF and extracts was taken as the control. The medium was removed at the end of incubation. Then, the 20 μL of 5 mg/mL 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) was added to each well, and incubation was continued for 4 h. Finally, 150 μL of dimethyl sulfoxide was added to each well and incubated for 10 min. The plate was read by a UV/vis spectrophotometer (Jasco model 7800, Japan) at 570 nm. The cell viability was calculated by dividing the absorbance of each experimental sample by the corresponding control sample.

NQO1 Activity Analysis. The activity of NQO1 was determined according to the method of Liu et al.[14] with slight modification. The single cell suspension was inoculated in 24-well microplates (2×10^5 cells/well in 1 mL of medium) and incubated with the SF solution (5 μM), the extracts from broccoli seeds through the HFE method (5 μM) and SHE method, respectively. The extracts through the SHE method were diluted by the same multiple with the HFE method. The solution without SF and extracts was used as the control. The mixture was cultured with 5% of CO_2 at 37 °C for 24 h. Cell suspension was then washed with PBS and digested with trypsin containing EDTA. The cell sediment after centrifugation at 2000*g* for 5 min was added to cell lysis buffer, dithiothreitol, and protease inhibitor (the ratio of 200:1:2) and disintegrated at 4 °C for 30 min. The supernatant was obtained by centrifugation at 12,000*g* for 30 min and stored at −80 °C. The supernatant was mixed with the reaction mixture [25 mM Tris-HCl buffer, 0.67 mg/mL bovine serum albumin (BSA), 0.01% Tween-20 (vol/vol), 0.03 mM $NADP^+$, 1 mM glucose-6-phosphate, 5 μM FAD, 2 U/mL glucose-6-phosphate dehydrogenase, 0.72 mM MTT, and 50 μM menadione (added immediately before reaction)] at a ratio of 1:4. The mixture was analyzed by a μQuant plate reader (BioTek, Winooski, VT, USA) and measured every 60 s over 7 min at 610 nm. The reaction was stopped by adding 50 μL of dicumarol (0.3 mM). The absorbance was measured for another 5 min to correct for the specific activity of non-NQO1. The protein concentrations were determined by the Bradford method with BSA as the standard.[28] The specific activity of NQO1 was defined as nM MTT reduced/min/mg protein.

Total ITC Quantification. The content of ITCs was measured by the cyclocondensation method through RP-HPLC according Liu et al.[14] In brief, the samples were incubated with potassium phosphate buffer (25 mM) and 1,2-benzenedithiol (10 mM) at 65 °C for 2 h. The mixture was centrifuged at 12,000*g* for 10 min after cooling to room temperature and measured by a C18 reverse-phase column (WondaCract ODS-2, 5 μm, 4.6 × 250 mm, Shimadzu, Japan) attached to an Agilent HPLC system (Agilent Technologies Inc., California, USA). The solvent system was operated with methanol (80%, vol/vol) and water (20%, vol/vol) in a flow rate of 1.0 mL/min at 365 nm. The standard was allyl isothiocyanate at a range of 12.5−200 μM.

GC−MS Analysis. The types of ITC analysis was measured by GC−MS and carried out according to the method of Wu et al.[25] with some modifications. A GC-MS 7890A (Agilent Technologies Inc., California, USA) equipped with Hp-5MS UI (0.25 μm ID × 30 m, injection volume 1 μL, Agilent Technologies Inc., California, USA) was used. The column temperature was 50−300 °C (50 °C for 2 min, 10 °C/min to 190 °C, 20 °C/min to 300 °C and was maintained for 5 min). The split ratio of carrier gas is 10:1. The mass spectrometry conditions were interface temperature 220 °C, ionization mode EI, ionization energy 70 eV, and mass range 35−500 amu. The GC was calibrated using standard curves of 1−100 mg/mL ITCs in dichloromethane. The cyclohexanone was used as the internal standard.

Statistical Analysis. Unless specifically mentioned, all the experiments were conducted as three replicates. Origin software (version 5.0) and SPSS statistics (version 17.0) were used for making standard deviations of the data.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c06319.

Mechanism erucin and sulforaphane hydrolyzed by myrosinase from glucoerucin and glucoraphanin; Sephadex G-75 gel filtration chromatogram of the crude extract of broccoli myrosinase; ITC production capacities of crude enzyme and each peak; and SDS-PAGE patterns of crude and purified broccoli myrosinase (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

3-BITC, 3-butenyl isothiocyanate; ESP, epithiospecifier protein; ER, erucin; ERN, erucin nitrile; HFE, hydrolysis followed by extraction; ITCs, isothiocyanates; NQO1, NAD(P)H:quinone oxidoreductase; NSP, nitrile-specifier proteins; SHE, simultaneous hydrolysis and extraction; PEITC, phenethyl isothiocyanate; SF, sulforaphane; SFN, sulforaphane nitrile; TFP, thiocyanate forming proteins

■ REFERENCES

(1) Ilahy, R.; Tlili, I.; Pék, Z.; Montefusco, A.; Siddiqui, M. W.; Homa, F.; Hdider, C.; R'him, T.; Helyes, L.; Lenucci, M. Pre- and post-harvest factors affecting glucosinolate content in broccoli. *Front. Nutr.* **2020**, *7*, 147.

(2) Favela-González, K. M.; Hernández-Almanza, A. Y.; Fuente-Salcido, N. M. D. L. The value of bioactive compounds of cruciferous vegetables (Brassica) as antimicrobials and antioxidants: A review. *J. Food Biochem.* **2020**, *44*, No. e13414.

(3) López-Chillón, M. T.; Carazo-Díaz, C.; Prieto-Merino, D.; Zafrilla, P.; Moreno, D. A.; Villaño, D. Effects of long-term consumption of broccoli sprouts on inflammatory markers in overweight subjects. *Clin. Nutr.* **2019**, *38*, 745−752.

(4) Raiola, A.; Errico, A.; Petruk, G.; Monti, D.; Barone, A.; Rigano, M. Bioactive compounds in brassicaceae vegetables with a role in the prevention of chronic diseases. *Molecules* **2017**, *23*, 15.

(5) Thomas-Charles, C.; Fennell, H. Anti-prostate cancer activity of plant-derived bioactive compounds: a review. *Curr. Mol. Biol. Rep.* **2019**, *5*, 140−151.

(6) Abukhabta, S.; Ghawi, S. K.; Karatzas, K. A.; Charalampopoulos, D.; McDougall, G.; Allwood, J. W.; Verrall, S.; Lavery, S.; Latimer, C.; Pourshahidi, L. K.; Lawther, R.; O'Connor, G.; Rowland, I.; Gill, C. I. R. Sulforaphane-enriched extracts from glucoraphanin-rich broccoli exert antimicrobial activity against gut pathogens in vitro and innovative cooking methods increase in vivo intestinal delivery of sulforaphane. *Eur. J. Nutr.* **2020**, 1.

(7) Yuanfeng, W.; Lv, C.; Zou, L.; Sun, J.; Song, X.; Zhao, Y.; Mao, J. Approaches for enhancing the stability and formation of sulforaphane-[J]. *Food Chem.* **2021**, *345*, 128771.

(8) Cieslik, E.; Leszczynska, T.; Filipiakflorkiewicz, A.; Sikora, E.; Pisulewski, P. Effects of some technological processes on glucosinolate contents in cruciferous vegetables. *Food Chem.* **2007**, *105*, 976−981.

(9) Clarke, J. D.; Hsu, A.; Riedl, K.; Bella, D.; Schwartz, S. J.; Stevens, J. F.; Ho, E. Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. *Pharmacol. Res.* **2011**, *64*, 456−463.

(10) Melchini, A.; Traka, M. Biological profile of erucin: a new promising anticancer agent from cruciferous vegetables. *Toxins* **2010**, *2*, 593−612.

(11) Citi, V.; Piragine, E.; Pagnotta, E.; Ugolini, L.; Di Cesare Mannelli, L.; Testai, L.; Ghelardini, C.; Lazzeri, L.; Calderone, V.; Martelli, A. Anticancer properties of erucin, an H_2S-releasing isothiocyanate, on human pancreatic adenocarcinoma cells (AsPC-1). *Phytother. Res.* **2019**, *33*, 845−855.

(12) Abbaoui, B.; Riedl, K. M.; Ralston, R. A.; Thomas-Ahner, J. M.; Schwartz, S. J.; Clinton, S. K.; Mortazavi, A. Inhibition of bladder cancer by broccoli isothiocyanates sulforaphane and erucin: Characterization, metabolism, and interconversion. *Mol. Nutr. Food Res.* **2012**, *56*, 1675−1687.

(13) Herz, C.; Hertrampf, A.; Zimmermann, S.; Stetter, N.; Wagner, M.; Kleinhans, C.; Erlacher, M.; Schüler, J.; Platz, S.; Rohn, S.; Mersch-Sundermann, V.; Lamy, E. The isothiocyanate erucin abrogates telomerase in hepatocellular carcinoma cells in vitro and in an orthotopic xenograft tumour model of HCC. *J. Cell. Mol. Med.* **2014**, *18*, 2393−2403.

(14) Liu, X.; Wang, Y.; Hoeflinger, J.; Neme, B.; Jeffery, E.; Miller, M. Dietary broccoli alters rat cecal microbiota to improve glucoraphanin hydrolysis to bioactive isothiocyanates. *Nutrients* **2017**, *9*, 262.

(15) Bheemreddy, R. M.; Jeffery, E. H. The metabolic fate of purified glucoraphanin in F344 rats. *J. Agric. Food Chem.* **2007**, *55*, 2861−2866.

(16) Saha, S.; Hollands, W.; Teucher, B.; Needs, P. W.; Narbad, A.; Ortori, C. A.; Barrett, D. A.; Rossiter, J. T.; Mithen, R. F.; Kroon, P. A. Isothiocyanate concentrations and interconversion of sulforaphane to erucin in human subjects after consumption of commercial frozen broccoli compared to fresh broccoli. *Mol. Nutr. Food Res.* **2012**, *56*, 1906−1916.

(17) Bricker, G. V.; Riedl, K. M.; Ralston, R. A.; Tober, K. L.; Oberyszyn, T. M.; Schwartz, S. J. Isothiocyanate metabolism, distribution, and interconversion in mice following consumption of thermally processed broccoli sprouts or purified sulforaphane. *Mol. Nutr. Food Res.* **2014**, *58*, 1991−2000.

(18) Iori, R.; Bernardi, R.; Gueyrard, D.; Rollin, P.; Palmieri, S. Formation of glucoraphanin by chemoselective oxidation of natural glucoerucin: A chemoenzymatic route to sulforaphane. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1047−1048.

(19) Vo, D.-V.; Van-Dat, T.; Thanh-Dao, T.; Van-Thanh-Nhan, D.; Ngoc-Tuan-Anh, P.; Khac-Minh, T. A new and effective approach to the synthesis of sulforaphane. *Lett. Org. Chem.* **2015**, *13*, 7−10.

(20) Pocasap, P.; Weerapreeyakul, N.; Thumanu, K. Alyssin and iberin in cruciferous vegetables exert anticancer activity in HepG2 by increasing intracellular reactive oxygen species and tubulin depolymerization. *Biomol. Ther.* **2019**, *27*, 540−552.

(21) Wang, N.; Wang, W.; Liu, C.; Jin, J.; Shao, B.; Shen, L. Inhibition of growth and induction of apoptosis in A549 cells by compounds from oxheart cabbage extract. *J. Sci. Food Agric.* **2016**, *96*, 3813−3820.

(22) García-Saldaña, J. S.; Campas-Baypoli, O. N.; Sánchez-Machado, D. I.; López-Cervantes, J. Separation and purification of sulforaphane (1-isothiocyanato-4-(methylsulfinyl) butane) from broccoli seeds by consecutive steps of adsorption-desorption-bleaching. *J. Food Eng.* **2018**, *237*, 162−170.

(23) Lv, X.; Meng, G.; Li, W.; Fan, D.; Wang, X.; Espinoza-Pinochet, C. A.; Cespedes-Acuña, C. L. Sulforaphane and its antioxidative effects in broccoli seeds and sprouts of different cultivars. *Food Chem.* **2020**, *316*, 126216.
(24) Tian, M.; Xu, X.; Hu, H.; Liu, Y.; Pan, S. Optimisation of enzymatic production of sulforaphane in broccoli sprouts and their total antioxidant activity at different growth and storage days. *J. Food Sci. Technol.* **2017**, *54*, 209−218.
(25) Wu, Y.; Shen, Y.; Wu, X.; Zhu, Y.; Mupunga, J.; Bao, W.; Huang, J.; Mao, J.; Liu, S.; You, Y. Hydrolysis before stir-frying increases the isothiocyanate content of broccoli. *J. Agric. Food Chem.* **2018**, *66*, 1509−1515.
(26) Sarvan, I.; Kramer, E.; Bouwmeester, H.; Dekker, M.; Verkerk, R. Sulforaphane formation and bioaccessibility are more affected by steaming time than meal composition during in vitro digestion of broccoli. *Food Chem.* **2017**, *214*, 580−586.
(27) Zhang, Y.; Xia, Y. J.; Ding, Z. Y.; Lai, P. F. H.; Wang, G. Q.; Xiong, Z. Q.; Liu, X. F.; Ai, L. Z. Purification and characteristics of a new milk-clotting enzyme from Bacillus licheniformis BL312. *LWT - Food Sci. Technol.* **2019**, *113*, 108276.
(28) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(29) Qarawi, M. A.; Carrington, S.; Blagbrough, I. S.; Moss, S. H.; Pouton, C. W. Optimization of the MTT assay for B16 murine melanoma cells and its application in assessing growth inhibition by polyamines and novel polyamine conjugates. *J. Pharm. Pharmacol.* **1997**, *3*, 235−239.
(30) Chiang, C.-C.; Huang, T.-N.; Lin, Y.-W.; Chen, K.-H.; Chiang, B.-H. Enhancement of 4-acetylantroquinonol B production by supplementation of its precursor during submerged fermentation of antrodia cinnamomea. *J. Agric. Food Chem.* **2013**, *61*, 9160−9165.
(31) Liang, H.; Yuan, Q.; Xiao, Q. Purification of sulforaphane from Brassica oleracea seed meal using low-pressure column chromatography. *J. Chromatogr., B* **2005**, *828*, 91−96.
(32) Liang, H.; Li, C.; Yuan, Q.; Vriesekoop, F. Separation and purification of sulforaphane from broccoli seeds by solid phase extraction and preparative high-performance liquid chromatography. *J. Agric. Food Chem.* **2007**, *55*, 8047−8053.
(33) Vaughn, S. F.; Berhow, M. A. Glucosinolate hydrolysis products from various plant sources: pH effects, isolation, and purification. *Ind. Crop. Prod.* **2005**, *21*, 193−202.
(34) Latté, K. P.; Appel, K.-E.; Lampen, A. Health benefits and possible risks of broccoli - An overview. *Food Chem. Toxicol.* **2011**, *49*, 3287−3309.
(35) Witzel, K.; Risha, M. A.; Albers, P.; Börnke, F.; Hanschen, F. S. Identification and characterization of three epithiospecifier protein isoforms in brassica oleracea. *Front. Plant Sci.* **2019**, *10*, 523.

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