JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Instability and Structural Change of 4-Methylsulfinyl-3-butenyl Isothiocyanate in the Hydrolytic Process

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ABSTRACT: Sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate), which has significant chemopreventive activities, is an important phytochemical ingredient produced by myrosinase hydrolysis of glucoraphenin (4-methylsulfinyl-3-butenyl glucosinolate) found in radish seeds. In this research, we found that sulforaphene was unstable and converted rapidly to a water-soluble degradation product in the hydrolytic process. The degradation product was successfully purified by preparative high-performance liquid chromatography on a C_{18} column using 10% methanol in water as the mobile phase. On the basis of MS and NMR spectroscopy data, the degradation product was identified to be 6-[(methylsulfinyl)methyl]-1,3-thiazinan-2-thione. The degradation pathway of sulforaphene was proposed in our study. Furthermore, low pH and metal ions were also found to have an effective inhibition to the degradation reaction of sulforaphene. Through adjusting the pH value of the system or adding metal ions after the content of sulforaphene has reached its maximum, the yield of sulforaphene increased significantly compared with that of the control.

KEYWORDS: *sulforaphene, glucoraphenin, radish seeds, degradation, 6-[(methylsulfinyl)methyl]-1,3-thiazinan-2-thione*

INTRODUCTION

Epidemiological investigations provide consistent evidence that cruciferous vegetables such as radish, broccoli, cabbage, Brussels sprouts and cauliflower have active ingredients in lowering the risk of developing lung cancer,[1,2] colorectal cancer,[3] breast cancer,[4] prostate cancer[5] and pancreatic cancer[6] in humans. Several studies revealed that these preventive effects appeared to be related to the significant amounts of diversified glucosinolates[7−9] and myrosinase (thioglucoside glucohydrolase, EC3.2.3.1) in cruciferous vegetables. After cruciferous vegetables have been chopped or ground, glucosinolates and myrosinase are brought into contact, and the β-thioglucoside bond of glucosinolates is hydrolyzed by myrosinase to produce sulfate compounds, glucose and a variety of aglycones. Subsequently, these aglycones undergo nonenzymatic and intramolecular rearrangement to yield thiocyanates, nitriles, isothiocyanates, epithionitriles and oxazolidine-thione.[9] The results of several pharmacological studies indicated that the cancer prevention activity of cruciferous vegetables would be strictly due to isothiocyanates, which is the most studied bioactive component hydrolyzed from glucosinolates.[10−14] However, natural isothiocyanate compounds are unstable in aqueous medium[15] and high temperature condition.[16] Furthermore, by virtue of their structure, isothiocyanates are strong electrophilic substances having a propensity to react with nucleophic groups, such as thiol, hydroxyl, and amino groups, to form dithiocarbamates, thiocarbamates, or thiourea derivatives.[17]

Among bioactive compounds in radish seeds,[18−22] sulforaphene (4-methylsufinyl-3-butenyl isothiocyanate), a member of isothiocyanate family derived from glucoraphenin (4-methylsulfinyl-3-butenyl glucosinolate), is strongly associated with cancer prevention.[23] As reported by Ippoushi and his co-workers, sulforaphene had cytotoxic and apoptotic activity on LoVo, HCT-116 and HT-29 human colon carcinoma cancer cell lines.[24] Additionally, sulforaphene has been shown to be capable of inhibiting the proliferative growth of human and murine erythroleukemic cells, human T-lymphoid cells, human cervix carcinoma cells and H3-T1−1 cells.[25] This research has demonstrated that sulforaphene possessed potential clinical utility as a chemopreventive substance. Moreover, sulforaphene possesses pharmacological antimutagenicity, antimicrobial and antiviral activities.[26−29] Therefore, it is important to establish an efficient method to obtain large amounts of sulforaphene with high purity for investigation of its chemopreventive activities. However, it was found that the yield of sulforaphene was much lower than the theoretical yield in our previous isolation process from radish seeds. So, it is believed that sulforaphene is unstable and easily degraded in the hydrolytic process of glucoraphenin. Our continuing studies in this area have led to the investigation of the degradation of sulforaphene in the hydrolytic process of glucoraphenin. At the same time, this study of hydrolysis condition has led us to maximize the yield of sulforaphene.

The objectives of this work are to study the degradation of sulforaphene, including identification of the degradation product, investigation of the degradation pathway, and searching for an efficient method to inhibit the degradation of sulforaphene during the hydrolysis process. By means of the structure of the degradation product, we proposed the degradation pathway of sulforaphene. Moreover, certain efficient methods were established based on the degradation pathway of sulforaphene to inhibit the degradation reaction and significantly increase the yield of sulforaphene.

Received: January 24, 2013
Revised: May 9, 2013
Accepted: May 10, 2013
Published: May 10, 2013

dx.doi.org/10.1021/jf400355d | *J. Agric. Food Chem.* 2013, 61, 5097−5102

EXPERIMENTAL SECTION

Materials and Methods. Radish seeds (yi tian bai yu xue) were purchased from Beijing Tongrentang Co., LTD (Beijing, China). Sulforaphene standard (>98%) was separated and purified from radish seeds by high-speed counter current chromatography (HSCCC) in our laboratory and its purity and chemical structure were identified by analytical HPLC, ESI-MS and NMR.[30] Methanol and trifluoracetic acid (TFA) used for analytical and preparative HPLC were of HPLC grade and purchased from Fisher Scientific Co., LTD (Tustin, CA). Ultra pure water was obtained by Q Millipore System (Millipore, Bedford, MA). The solvents for analytical HPLC and Preparative HPLC were filtered with 0.45 μm membrane and degassed by ultrasound for 30 min before use. KH_2PO_4, K_2HPO_4, Na_2S, HCl, $ZnCl_2$, $CuCl_2$, $CaCl_2$, $MgCl_2$, $FeCl_2$, and $FeCl_3$ were analytical grade and purchased from Beijing Chemical Works (Beijing, China).

The analytical HPLC equipment used in the analysis experiment was a Shimadzu LC-20AT system with two LC-20AT solvent delivery units, a SPD-M20A DAD detector, a SIL-20A auto sampler, a CTO-10ASVP column oven, a LC solution workstation (Shimadzu, Kyoto, Japan) and an analytical reversed phase C_{18} column (4.6 × 250 mm, 5 μm, Diamodsil). The preparative HPLC equipment used in the preparation experiment was a Waters Prep 4000 liquid chromatography system equipped with a fluid handling unit (pump heads), controller (for solvent gradient, flow rate, external events, and sparging process), a 2487 dual-wavelength absorbance detector with a preparative cell (Waters, Milford, MA), an Empower workstation (Waters) and a preparative reversed phase C_{18} column (19 × 300 mm, 7 μm, Symmetry Prep). An ESI-MS spectrum of the purified degradation product was obtained on a high resolution mass spectrometer with an ion source. NMR (^{1}H, ^{13}C, DEPT-135, COSY, HSQC, HMBC) spectrum of the purified degradation product were obtained on a Bruker high-resolution AV600 NMR spectrometer at 600 MHz and DMSO-d_6 was used as solvent with 0.03% TMA (v/v) as internal standard.

The Degradation of Sulforaphene. Two grams of radish seeds were ground into powder with an analytical grinder and then added into the 20 mL phosphate buffer solution (pH = 7.0, 20 mM). The mixture of seed powder and buffer solution was allowed to spontaneously autolyze for different time intervals at 25 °C. The mixture were centrifuged at 12000 rpm/min for 5 min and filtered with 0.22 μm of membrane. Then the formation of sulforaphene and its degradation product in the mixture were analyzed by analytical HPLC.

HPLC Analysis. Sulforaphene and its degradation product were separated by eluting with a gradient of methanol (mobile phase A) and 0.02% (v/v) TFA aqueous solution (mobile phase B) as follows: linear gradient from 5% A to 80% A for 30 min, 100% A kept for 2 min to purge the column, then 5% A kept for 8 min to equilibrate the column. The injection volume was 10 μL portion per sample, the flow rate was 1 mL/min, the detection wavelength was set at 254 and 281 nm with a resolution of 1.2 nm, and the temperature of the column oven was set at 30 °C.

Separation of the Degradation Product. Sulforaphene (300 mg) with the purity of 98% and 2 g radish seeds which were ground into powder with an analytical grinder were added into 20 mL phosphate buffer solution (20 mM, pH = 7.0), and the mixture was allowed to spontaneously autolyze for 12 h at 25 °C to produce the degradation product. After being centrifuged at 12000 rpm/min for 5 min and filtrated with 0.22 μm of membrane, the degradation product-rich extract was subjected into the preparative HPLC system. The preparative HPLC separation was performed as follows: the mobile phase system consisted of 10% (v/v) methanol in ultrapure water, the flow rate was 10 mL/min, the column temperature was set at 30 °C, the detection wavelength was set at 281 nm, and the injection volume was 1 mL. The peak of the degradation product was collected manually according to the preparative HPLC chromatogram.

Identification of the Degradation Product. The identification of the degradation product purified by preparative HPLC was carried out by MS and NMR (^{1}H, ^{13}C, DEPT-135, COSY, HSQC, HMBC). A High resolution mass spectrometer was used with an ion source temperature of 200 °C and a probe temperature of 25 °C. The MS spectrum was scanned at 25 eV from m/z: 50−500. Five milligrams of purified degradation product was dissolved into 500 μL of DMSO-d_6 with 0.03% TMS (v/v) as internal standard and then subjected to a Bruker high-resolution AV600 NMR spectrometer at 600 MHz.

The Proposed Degradation Pathway of Sulforaphene. HCl (6 mol/L, 50 mL) was dripped into Na_2S (25 g) to produce H_2S and then the gas was passed into 50 mL phosphate buffer solution (20 mM, pH = 7.0) containing pure sulforaphene (50 mg). After 30 min, the sample was taken from the reaction system and subjected to analytical HPLC to analyze the product. Based on the same method, the sample from the acidic reaction system (pH = 2.0) was also analyzed by analytical HPLC.

The Effect of pH Value on the Degradation Reaction. Two grams of radish seeds ground into powder with an analytical grinder were added into 20 mL phosphate buffer solution (20 mM, pH = 7.0) and the mixture was allowed to spontaneously autolyze for 20 min at 25 °C. Then the pH value of the hydrolysis system was adjusted to 1.0 with HCl. After 4 h, a sample of the mixture was subjected into HPLC analysis system to analyze the contents of sulforaphene and its degradation product. According to the method described above, the pH value of the system was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0 with HCl (6 mol/L) and 8.0, 9.0, 10.0 with NaOH (3 mol/L). Samples of different pH value were subjected to the analytical HPLC to analyze the contents of sulforaphene and its degradation product.

Effects of Different Metal Ions on the Degradation Reaction. Two grams of radish seeds ground into powder with an analytical grinder were added into 20 mL phosphate buffer solution (20 mM, pH = 7.0) and the mixture was allowed to spontaneously autolyze for 20 min at 25 °C. Then 10 mM metal ion (Zn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}) was added into the hydrolysis system. Samples of different time intervals were obtained and subjected to analytical HPLC to analyze the contents of sulforaphene and its degradation product.

RESULTS AND DISCUSSION

HPLC Analysis the Degradation of Sulforaphene. From Figure 1A, most of sulforaphene degraded after 120 min compared with its initial content at 20 min in the hydrolysis system. As shown in Figure 1B, when sulforaphene began to decrease, a new peak at 281 nm started to appear and increased about 20-fold from 20 to 120 min. Based on the chromatogram of HPLC shown in Figure 1B, we speculated the new peak was the degradation product of sulforaphene. To confirm that the generation of the degradation product originated from sulforaphene, about 40 mg of fresh sulforaphene with high purity was added into the system when sulforaphene completely converted to its degradation product in the hydrolysis system. From Figure 2B, the progressive loss of pure sulforaphene was accompanied by a respective increase in the content of the degradation product from 120 to 240 min. As compared with the result of the control experiment without adding pure sulforaphene (Figure 2A), the increased degradation product completely derived from the pure sulforaphene added in the hydrolysis system at 120 min. Our finding confirmed that this compound derived from the degradation of sulforaphene.

Separation the Degradation Product by Preparative HPLC. The degradation product with high purity was obtained by separation and purification method for identifying its structure. In the purification experiment, we found that using 10% methanol in water as the mobile phase was ideal for the degradation product. According to the chromatogram of preparative HPLC shown in Figure 3, the major peak (the fraction from 45 to 48 min) was collected and then the obtained eluent was subjected to analytical HPLC to analyze. As it was shown in Figure 3, the obtained eluent contained the degradation product. After freeze-drying, the degradation product was obtained as colorless needles. The degradation product was further analyzed by a 3D HPLC chromatogram obtained by the

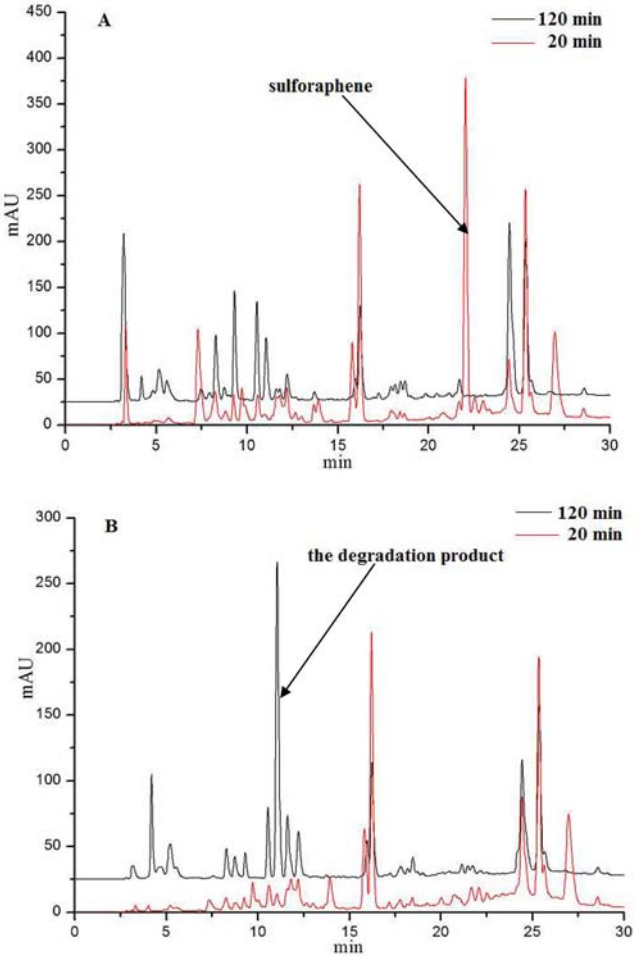

Figure 1. Chromatograms of the analysis HPLC of sulforaphene and its degradation product. (A) Change in content of sulforaphene in hydrolysis system at 20 min, 120 min (maximum absorption wavelength at 254 nm). (B) Change in content of degradation product in hydrolysis system at 20 min, 120 min ((maximum absorption wavelength at 281 nm).

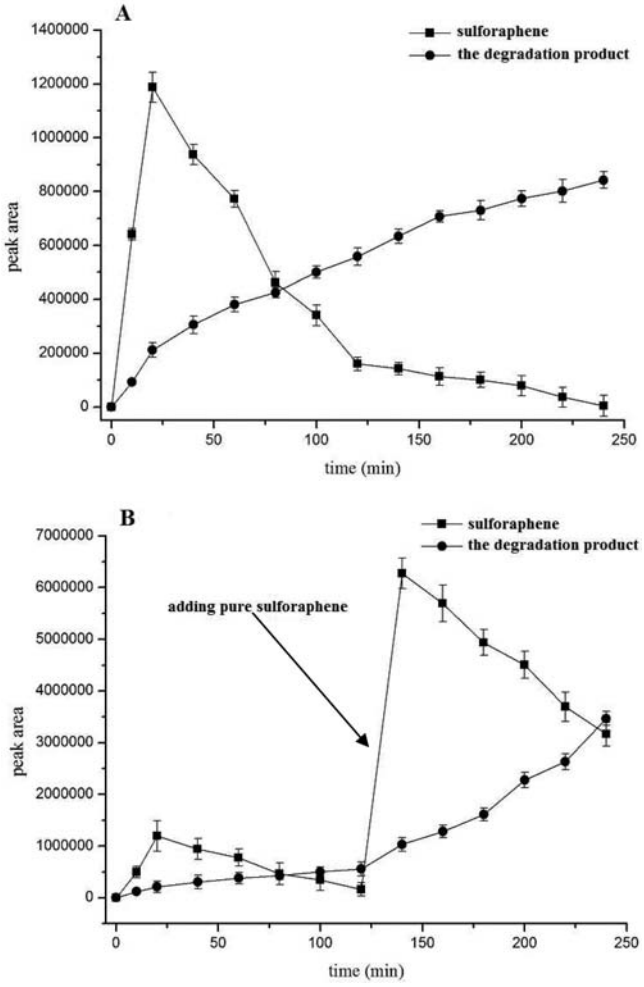

Figure 2. (A) Contents of sulforaphene and its degradation product in the hydrolysis system. (B) Contents of sulforaphene and its degradation product after adding pure sulforaphene. The experiment was carried out in triplicate. The data were expressed as means ± standard deviations (SD).

diode array detector and the purity of it was found to be more than 98%.

Identification of the Degradation Product. The structure of the degradation product was identified by MS and NMR. The HR-ESI-MS spectrum showed the degradation product has a molecular formula of $[C_6H_{12}NOS_3]^+$ (calculated: 210.0081, found: 210.0085). Compositions of $[C_5H_8NS_2]^+$ (calculated: 146.0098, found: 146.0103), $[C_3H_4NS_2]^+$ (calculated:117.9785, found: 117.9787), $[C_4H_7S]^+$ (calculated: 87.0268, found: 87.0265) for MS/MS fragment ions also supported the analysis of the structure of the degradation product. The ^1H and ^{13}C NMR data, as well as the correlations in COSY, HSQC and HMBC spectrum of the degradation product were listed in Table 1. Combined with the DEPT-135 spectrum, it could be indicated that C-4, C-5 and C-7 were secondary carbons and C-2 was a quaternary carbon. From ^1H and HSQC spectrum, C-6 and C-8 were further confirmed as tertiary carbon and primary carbon respectively. In addition, all the three secondary carbons (C-4, C-5, C-7) were shown directly bonded with two inequivalent protons, which was also confirmed by $^1H-^1H$ COSY spectrum with the correlations (cross peaks, δ 1.82−1.89 and δ 2.28−2.33, δ 2.94 and δ 3.15, δ 3.31−3.36 and δ 3.42−3.47, as shown in Table 1). According to the HMBC spectrum, the correlations of C-8 with the two inequivalent protons (δ 2.94, δ 3.15) indicated that the cyclization reaction was located at C-6 of the degradation product. In summary, based on the MS, MS/MS and NMR data analysis, the structure of the degradation product was identified as 6-[(methylsulfinyl) methyl]-1,3-thiazinan-2-thione and its structure is shown in Figure 5.

The Proposed Degradation Pathway of Sulforaphene. From the molecular formula of sulforaphene and the degradation product, a sulfur atom difference was found between them, so a sulfur compound was involved in the reaction. Some reports showed that cruciferous vegetables produce many volatile sulfur compounds upon tissue disruption, such as hydrogen sulfide and methanethiol-related compounds.[31,32] From Figure 4, we found that sulforaphene degraded and converted completely to the degradation product when H_2S passed through the aqueous at neutral condition compared with that of the control. On the basis of the aforementioned finding, the degradation pathway of sulforaphene was proposed. As shown in Figure 5, the thiocyanate group of sulforaphene was strong electrophilic and easily reacted with H_2S, and dithiocarbamate formed as an intermediate product in the hydrolysis system. The nucleophilic SH group attacked the carbon atom involved in the double bond, followed by the internal cyclization of the resulting intermediate

dx.doi.org/10.1021/jf400355d | J. Agric. Food Chem. 2013, 61, 5097−5102

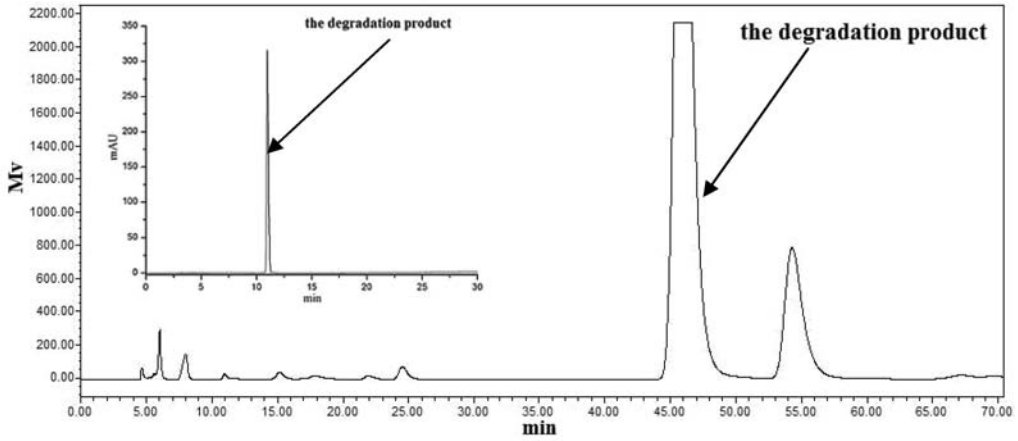

Figure 3. Preparative HPLC chromatogram of the degradation product and the analytical HPLC chromatogram of the purified degradation product obtained by preparative HPLC.

Table 1. NMR Data Used for Elucidating the Structure of the Degradation Product

position	δ H (δ/ppm; J/Hz)	DEPT 135	δ C (δ/ppm)	HSQC (H−C)	COSY (H−H)	HMBC (H−C)
2		C	190.9			
3	10.41(1H, s)					
4	3.42−3.47(1H, m); 3.31−3.36(1H, m)	CH_2	42.2	C_4	H_4, H_5	C_2, C_5, C_6
				C_4	H_4, H_5	C_2, C_5, C_6
5	1.82−1.89(1H, m); 2.28−2.33(1H, m)	CH_2	25.6	C_5	H_4, H_5, H_6	C_4, C_6, C_7
				C_5	H_4, H_5, H_6	C_4, C_6, C_7
6	3.72−3.77(1H, m)	CH	38.5	C_6	H_5, H_7	C_2, C_4, C_5, C_7
7	2.94(1H, dd, 13.2, 4.8); 3.15(1H, dd, 13.2, 9.3)	CH_2	57.3	C_7	H_6, H_7	C_5, C_6, C_8
				C_7	H_6, H_7	C_5, C_6, C_8
8	2.62(3H, s)	CH_3	38.4	C_8		C_7

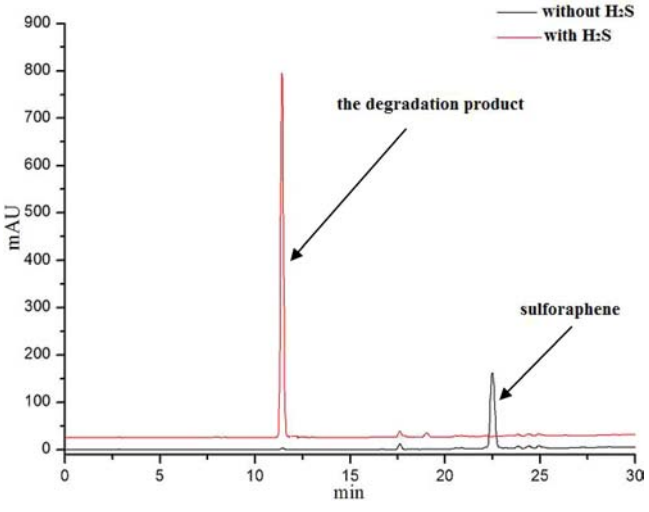

Figure 4. Chromatogram of the analysis HPLC of sulforaphene (without H_2S) and its degradation product (with H_2S) at 254 nm.

to form the cyclic degradation product. Since H_2S is a weak nucleophile in low pH medium, the reaction between sulforaphene and H_2S is negligible. In the degradation pathway of sulforaphene, the pH value of the medium plays an important role between sulforaphene and H_2S.

Inhibition of the Degradation Reaction with HCl. Sulforaphene produced by glucoraphenin degraded rapidly to form the degradation product. As a result, the yield of sulforaphene was low. Therefore, a search of an efficient method to inhibit the degradation reaction of sulforaphene is pressingly demanded. The pH value of the hydrolysis system was important for the hydrolytic reaction and had direct effect on the yield of the isothiocyanates.[33] It was reported that the isothiocyanates easily formed at pH equal to 7.0 in the breakdown process of glucosinolates by myrosinase.[9] Then the hydrolytic process from glucoraphenin to sulforaphene should be kept under neutral condition. Our work has found that the degradation reaction could be inhibited under acid condition. Encouraged by our findings, we sought to ascertain a minimization of the formation of degradation product by employing different pH values in the medium during the hydrolysis. We began by keeping pH at 7.0 in the first 20 min, and then changed the pH value during the course of hydrolysis. After 4 h, the content of sulforaphene was significantly higher when the pH of the hydrolysis system was adjusted to 2.0 at 20 min than its content at other pH values. At the same time, the content of the degradation product of sulforaphene was also less than those at other pH values (Figure 6). It is noteworthy that sulforaphene would degrade and convert to the degradation product completely in alkaline condition in 4 h. We conclude that adjusting the pH value of the hydrolysis system to 2.0 with HCl could effectively inhibit the degradation when the content of sulforaphene reached its maximum. Through this method, the content of sulforaphene was improved about 26.1 times compared with that of neutral condition (shown in Figure 6).

Inhibition of the Degradation Reaction with Metal Ions. Effects of six metal ions (Cu^{2+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Zn^{2+}) on the degradation reaction were also investigated in our research. Chlorides of metal ions were used in the hydrolysis

dx.doi.org/10.1021/jf400355d | J. Agric. Food Chem. 2013, 61, 5097−5102

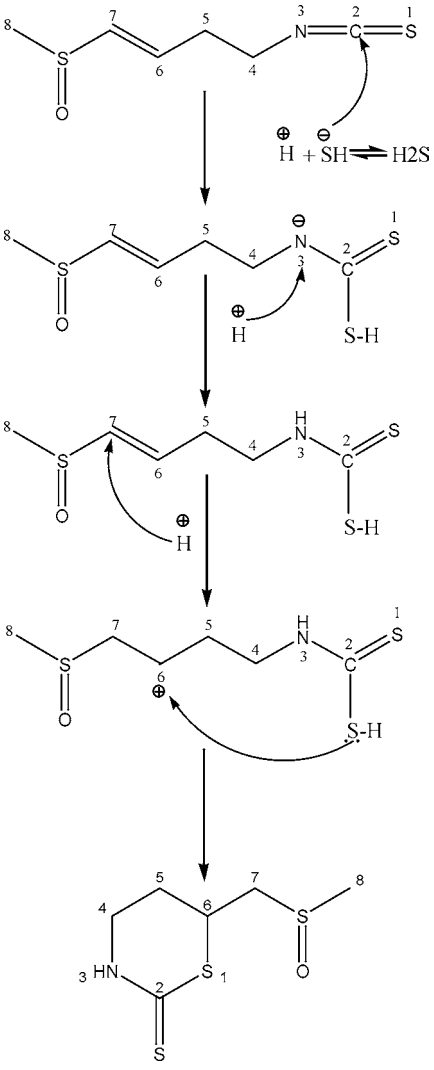

Figure 5. Proposed degradation pathway of sulforaphene.

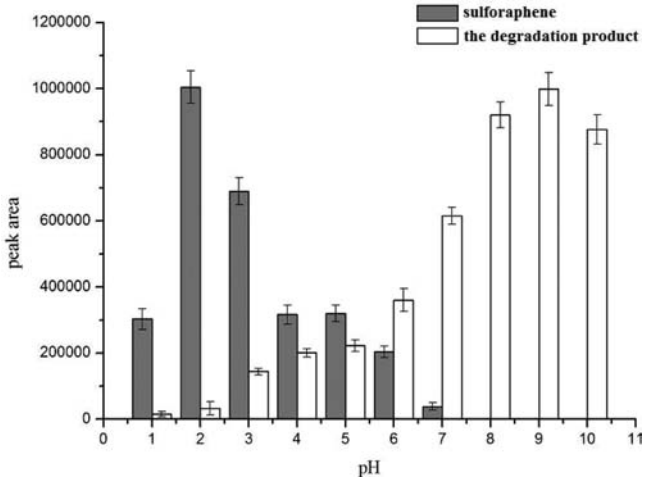

Figure 6. Contents of sulforaphene and its degradation product in the hydrolysis system of different pH value. The experiment was carried out in triplicate. The data were expressed as means ± standard deviations (SD).

system to remove H_2S produced by radish seeds. As is shown in Figure 7, all selected metal ions could inhibit the degradation

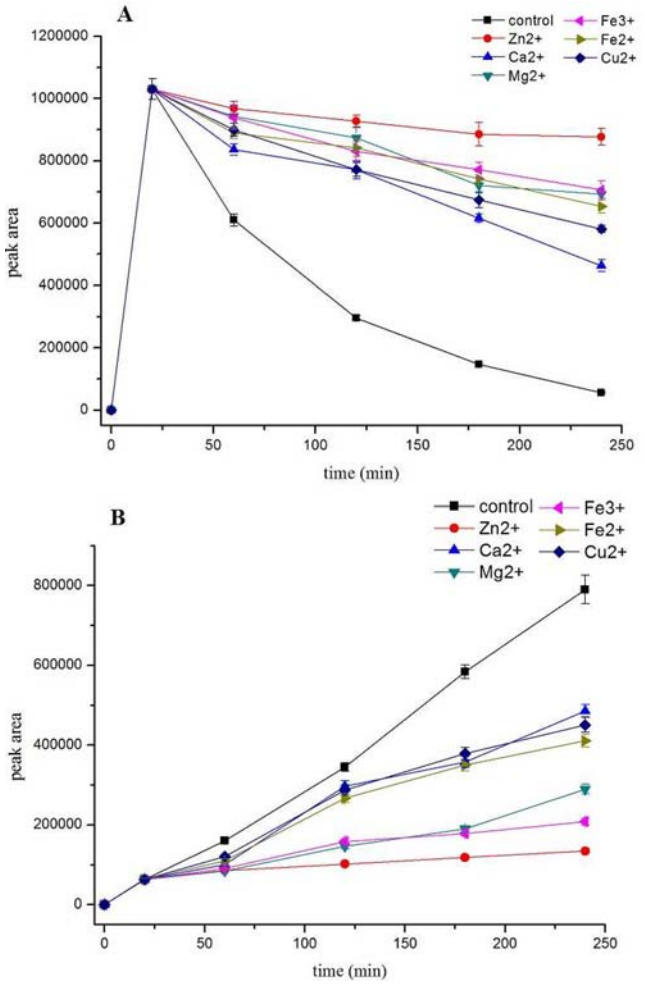

Figure 7. (A) Content of sulforaphene in the hydrolysis system after adding different metal ions. (B) Content of the degradation product in the hydrolysis system after adding different metal ions. The experiment was carried out in triplicate. The data were expressed as means ± standard deviations (SD).

reaction in different levels. Compared with the reaction system without metal ions, the productivity of sulforaphene increased after adding all selected metal ions into the hydrolysis system. All selected metal ions could react with H_2S and form insoluble precipitate to remove H_2S in the hydrolysis system, and the reaction between sulforaphene and H_2S was inhibited. Therefore, metal ions were also important for the degradation reaction of sulforaphene.

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Author Contributions

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge financial support from the Natural Science Foundation of China (20806005, 21176018) and the National

High Technology Research and Development Program of China (863 Program, Grant No. 2012AA021403).

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