Food Sci. Biotechnol. Vol. 15, No. 5, pp. 688 ~ 693 (2006)

Food Science and Biotechnology

Changes in Isothiocyanate Levels in Korean Chinese Cabbage Leaves during *Kimchi* Storage

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Abstract Glucosinolates are hydrolyzed by the enzyme myrosinase and are mainly found in cruciferous vegetables such as Chinese cabbage *(Brassica campestris* L. ssp. *pekinensis*). Isothiocyanates (ITCs) are glucosinolate degradation products with reported anticarcinogenic properties. Korean Chinese cabbage in the form of '*kimchi*' is a staple part of the Korean diet. In this study, we examined the effects of storage temperature and duration on glucosinolate, ITC, soluble sugar, and organic acid levels in *kimchi*. Changes in pH and the impact of various parts of the Korean Chinese cabbage being used during the preparation of the dish were also assessed. Extracted ITC levels, analyzed via gas chromatography (GC) and GC/mass spectrometry (GC/MS), were higher in the midrib parts than in the cabbage leaves after storage at both 4 and 20°C. During storage, organic acid levels increased while soluble sugars were depleted. The pH initially increased (after 1 day at 20°C, and 1 week at 4°C), but subsequently decreased over time at both temperatures. Glucosinolate and ITC levels increased in the beginning of storage but then generally fell during further storage. Our data suggest that acidity-related reduction in myrosinase activity during storage may decrease glucosinolate and ITC levels. The changes in these levels depended on the storage conditions and the Korean Chinese cabbage parts used for the *kimchi* preparation.

Keywords: *kimchi*, Korean Chinese cabbage, storage, total glucosinolates, isothiocyanates

Introduction

Epidemiological studies associate the consumption of cruciferous vegetables with a decreased risk of cancer, but the underlying mechanism is uncertain (1, 2). Vegetables like broccoli, cauliflower, brussel sprouts, cabbage, turnip, and horseradish are rich in sulphur-containing glucosinolates originating from protein and non-protein metabolism (3, 4). When plant tissue is damaged, the enzyme myrosinase (β-thioglucosidase, EC 3.2.3.1) hydrolyzes glucosinolates (e.g., β-D-thioglucosides) to glucose, sulfate, isothiocyanates (ITCs), nitrile, thiocyanate, etc (5, 6). Glucosinolate metabolites have been reported to protect against lung, colon, liver, and stomach cancer (7). In particular, ITCs - components of a plant's natural defense system against insects, fungi, and microbial infections (10) - appear to have important anticarcinogenic activity (8, 9).

Chinese cabbage is a major food source in Asian countries. Particularly common is the Korean '*baechukimchi*' ('*kimchi*'), a traditional fermented vegetable dish served with red pepper, garlic, ginger, and a fish source. *Kimchi* and its antimutagenic/anticarcinogenic potential have been intensely researched (11, 12) with results demonstrating that optimally fermented *kimchi* was more effective than freshly prepared *kimchi* (13). To date, changes in the glucosinolate and ITC content during *kimchi* storage have not been sufficiently examined. The objective of this study was to measure the glucosinolate and ITC levels (e.g., 3-butenyl, 4-pentenyl and ß-phenylethyl ITC) during storage at 20 and 4°C.

Materials and Methods

Preparation of *kimchi* Cultivars of 'Noraengi' Korean Chinese cabbage were purchased at the Garak agricultural market in Seoul, Korea. *Kimchi* was prepared according to the standard recipe of the Korean Rural Development Administration. Briefly, 22 kg of cabbage was soaked in 10% NaCl for 2 hr at room temperature. The salted cabbage was to reach 2% salt concentration. The salted cabbage was subsequently mixed with powdered red pepper (990 g), garlic (440 g), ginger (198 g), *jeotkal* (salted-fermented seafood) (1,100 g), sesame (110 g), commercial sugar (110 g), and glutinous rice paste (1,870 g). The mixture was divided into individual (1 kg), tightly-closed jars and stored at 20°C for up to 1 week or at 4°C for up to 4 weeks.

Chemicals All reagents were laboratory grade and were purchased from Junsei Chemical Co. (Tokyo, Japan). Standard solutions of 3-butenyl ITC, 4-pentenyl ITC, and 2-phenylethyl ITC were purchased from Kasei (Tokyo, Japan).

Preparation of crude myrosinase Myrosinase was prepared from locally purchased radish. The roots of the vegetable were chilled, homogenized in a blender, and filtered through two layers of gauze. One and a half volumes of acetone were added (4°C) to the juice and the mixture was left on ice for 5 min. Following centrifugation (1,864×g for 15 min at 4°C), the precipitate containing the crude enzyme was freeze-dried, ground into powder, and stored at -20°C prior to its use.

Determination of pH The pH of the *kimchi* liquid was measured periodically during storage at 4 and 20°C.

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Received February 24, 2006; accepted August 29, 2006

Sample preparation for organic acid and soluble sugar measurements To measure the organic acid and soluble sugar contents of the *kimchi*, the seasoning was removed and 10 g samples were homogenized in 30 mL of distilled water. Each sample was shaken at 75×g for 30 min, centrifuged at 26,895×g at 4°C for 22 min, and the supernatant was filtered through 0.45 μm membrane filters.

Quantitative organic acid analysis Organic acid content was determined with high pressure liquid chromatography (HPLC) using an LC30 chromatography oven and an ED40 electrochemical detector linked to a GP40 gradient pump (Younglin Instrument, Korea) and a column fitted with Ionpac® (Dionex, Sunnyvale, CA, USA). Nanopure water was used for all HPLC assays (Millipore Milli Q Plus). Tetrabutylammonium hydroxide (0.4 N) and heptafluorobutyric acid (0.4 mM) were used as the mobile phase at a flow rate of 0.8 mL/min at 30°C.

Quantitative soluble sugar analysis Soluble sugar content was determined via HPLC using a CTS-30 oven, RI detectors linked to an SP-930D pump (Younglin Instrument), and a column (6.5×300 mm) fitted with a Sugar-Pak (Waters, Millipore, MA, USA) at 70°C. Nanopure water was used for all HPLC assays. Distilled water was used as the mobile phase at a flow rate of 0.5 mL/min.

Sample preparation for measurements of total glucosinolate and isothiocyanate content The *kimchi* was removed from the seasoning mixture and divided into midribs and leaves. Fifty g samples of each were added to 100 mL of hot 80% ethanol and the mixture was boiled for 15 min in a water bath and then homogenized with a blender. After repeating these initial steps, the extract was filtered and condensed to 25 mL with an evaporator (EYELA, Tokyo, Japan) at 40°C. The samples were then centrifuged at 1,864×g at 4-5°C for 15 min and the supernatant was adjusted to 50 mL with distilled water. A 25 mL aliquot was passed through an anion exchange column (5 mL of Dowex 1-X^2, Cl$^-$ form, 50/100 mesh). The column was washed with 50 mL water (equal to ten volumes of the 5 mL anion exchange resin) until no further glucose reaction was detectable via Molisch reagent analysis. The ion exchange resin was transferred into a 50 mL Erlenmeyer flask containing 5 mL CH_2Cl_2, 50 mg crude myrosinase, 1 mL ascorbic acid (10 mM), and 5 mL Na_2PO_4 buffer (0.1 M, pH 7.0). After gently shaking the flask at room temperature for 18 hr, the enzyme mixture was centrifuged at 1,864×g for 15 min at 4°C. The methylenechloride layer (bottom part of the mixture in the centrifuge tube) was used for ITC gas chromatography (GC) analysis. The water layer (upper part) was used to determine the glucosinolate content.

Measurement of glucosinolate and isothiocyanate content The glucosinolate content was assessed with a spectronic Unicam spectrophotometer (Model Genesis 10vis; Cambridge, UK) at 505 nm using the thymol method (14). Sample solution, thymol reagent, and H_2SO_4 were boiled in a water bath for 35 min. A glucose standard calibration curve was used to quantify the total glucosinolate concentration. The conditions for the GC and GC/mass spectrometry (GC/MS) analysis of ITCs are shown in Table 1 and 2.

Results and Discussion

pH of *kimchi* during storage The pH of the *kimchi* was measured periodically during the storage period. It is well established that *kimchi* ripens more rapidly at higher temperatures with a resultant significant acidification to pH 3.0-5.0 (15). As shown in Fig. 1, the pH dropped sharply when the *kimchi* was stored at 20°C, but only slowly at 4°C. The pH also fell markedly between 1-3 days of storage at 20°C and between 1-2 weeks at 4°C, reflecting the characteristic drop in pH during the initial storage period for *kimchi*. Previous studies have found that initial pH values for *kimchi* (pH 5.5-5.8) fall to pH 4.2-4.5 at optimum ripening, and to pH 4.0 upon over-ripening (16).

Organic acid and soluble sugar content The organic acid content of *kimchi* during storage at 20 and 4°C is listed in Table 3. Changes in organic acid levels are shown in Fig. 2. Lactic and succinic acid levels increased during storage at 20°C. At 20°C, the organic acid level was higher

Table 1. GC conditions for isothiocyanate measurements

Instrument	Agilent GC 4890
Column	DB-5, 30 m, i.d. 0.53 mm, 0.5 μm
Carrier gas	N_2, 1 mL/min
Split	30:1
Detector	FID
Inlet temperature	280°C
Detector temperature	280°C
Oven temperature	80°C, 1 min 80 - 180°C (8°C/min) 180 - 255°C (30°C/min)
Injection volume	2 μL

Table 2. GC/MS conditions for isothiocyanate measurements

Instrument	Agilent GC 6890, Agilent MSD 5973
Column	Ultra 25% phenyl methyl siloxane (19091B-005 : 0.20 mm × 50 m)
Carrier gas	H_2
Split	10:1
Detector	FID
Injector temperature	280°C
Detector temperature	280°C
Oven temperature	80°C, 5 min 80 - 180°C (8°C/min) 180 - 255°C (30°C/min)
Injection volume	1 μL

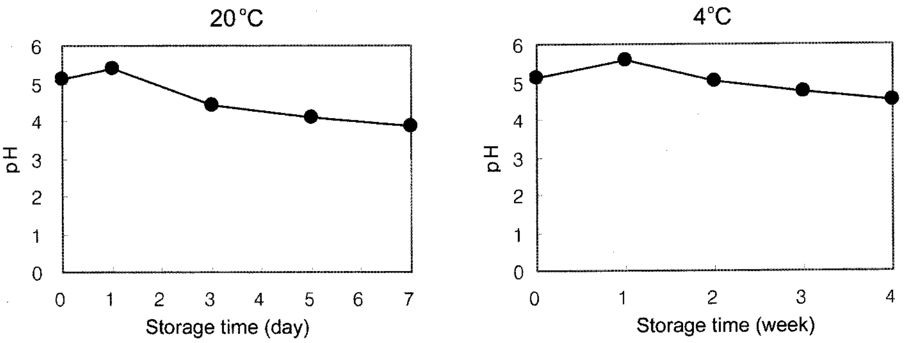

Fig. 1. pH changes during *kimchi* storage.

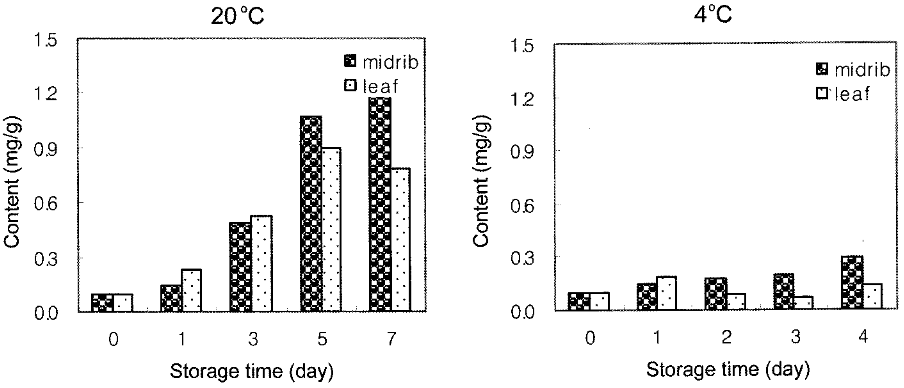

Fig. 2. Organic acid levels during *kimchi* storage.

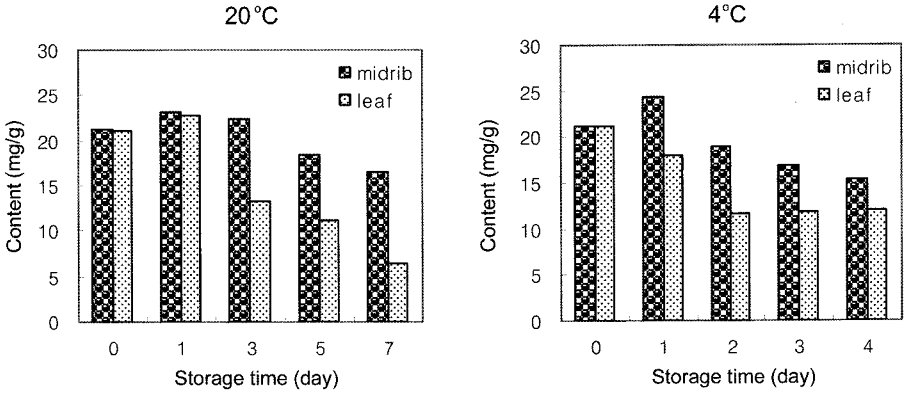

Fig. 3. Soluble sugar content during *kimchi* storage.

than at 4°C. It has been reported that *kimchi* stored at higher temperature contains more organic acid than low-temperature stored controls (17).

The soluble sugar content of *kimchi* during storage at 20 and 4°C is listed in Table 4 and graphically plotted in Fig. 3. Soluble sugars, in particular glucose and fructose, generally decreased over time. These results show that microorganisms present in the *kimchi* used the reducing sugars of the cabbage as an energy source. Furthermore, the organic acid and soluble sugar levels in the midrib were higher than those in the leaf.

Glucosinolate content The glucosinolate content was determined by the thymol method (14). Figure 4 shows the changes in glucosinolate levels during *kimchi* storage. At 20°C, glucosinolates increased during the first day, but slowly decreased thereafter. Midrib glucosinolate content ranged between 90.41 and 121.26 μmol/100 g, compared to 79.49-107.58 mmol/100 g in the leaves.

At 4°C, glucosinolate levels increased during the first week of storage, but gradually declined during the following three weeks. Midrib glucosinolate content ranged between 98.17 and 137.06 μmol/100 g. The leaves had comparable glucosinolate levels (98.31-154.25 μmol/100 g).

Glucosinolate levels were comparatively higher after one week of storage at 4°C than at 20°C, demonstrating the importance of storage temperature for glucosinolate content. It was also noted that the midribs had a higher glucosinolate concentration than the leaves. In contrast,

Table 3. Organic acid content (mg/g) during *kimchi* storage

20°C		Tartaric acid	Malic acid	Lactic acid	Succinic acid
0 day	midrib	0.01	0.07	-[1]	0.02
	leaf	0.03	0.02	0.02	0.03
1 day	midrib	0.03	0.07	0.01	0.02
	leaf	0.09	0.09	0.03	0.02
3 day	midrib	0.02	0.05	0.30	0.12
	leaf	0.02	0.01	0.38	0.12
5 day	midrib	0.02	0.01	0.77	0.28
	leaf	0.02	0.01	0.70	0.17
7 day	midrib	0.02	-	0.92	0.25
	leaf	0.01	-	0.68	0.09

4°C		Tartaric acid	Malic acid	Lactic acid	Succinic acid
0 week	midrib	0.01	0.07	-	0.02
	leaf	0.03	0.13	0.02	0.03
1 week	midrib	0.04	0.08	0.01	0.01
	leaf	0.09	0.07	0.02	0.01
2 week	midrib	0.07	0.08	0.02	0.01
	leaf	0.06	0.02	0.01	-
3 week	midrib	0.08	0.08	0.03	0.01
	leaf	0.04	0.01	0.01	-
4 week	midrib	0.09	0.06	0.12	0.02
	leaf	0.06	0.01	0.06	-

[1]- : Not detected.

Table 4. Soluble sugar content (mg/g) during *kimchi* storage

20°C		Sucrose	Glucose	Fructose	Mannitol
0 day	midrib	-[1]	11.49	9.75	-
	leaf	1.23	11.52	8.39	-
1 day	midrib	1.03	11.97	10.11	-
	leaf	1.44	12.10	9.32	-
3 day	midrib	1.16	10.04	8.07	3.07
	leaf	1.24	5.15	3.49	3.35
5 day	midrib	1.12	6.41	2.69	8.17
	leaf	1.41	3.53	1.33	4.93
7 day	midrib	1.07	5.41	2.11	7.88
	leaf	1.23	1.29	-	3.98

4°C		Sucrose	Glucose	Fructose	Mannitol
0 week	midrib	-	11.49	9.75	-
	leaf	1.23	11.52	8.39	-
1 week	midrib	1.54	13.26	9.61	-
	leaf	1.59	9.27	7.15	-
2 week	midrib	1.80	10.47	6.57	-
	leaf	1.42	5.90	4.22	-
3 week	midrib	1.99	8.73	6.20	-
	leaf	1.45	6.00	4.39	-
4 week	midrib	1.99	7.55	5.88	-
	leaf	1.34	6.10	4.58	-

[1]- : Not detected.

fresh Korean Chinese cabbage had higher glucosinolate levels in the leaves (midrib, 119.65 μmol/100 g; leaves, 148.77 μmol/100 g). These results were compared to the change in pH during storage of *kimchi.* In an earlier study, it was reported that glucosinolates were unstable in acidic buffer (18). It is conceivable that the gradual decrease in total glucosinolates during *kimchi* storage is related to the associated pH drop.

Isothiocyanate content ITCs (3-butenly, 4-pentenyl and β-phenylethyl) in the *kimchi* extracts were determined by GC/MS (Fig. 5-7). ITC content in the extracts was the highest for 4-pentenyl ITC, followed by 3-butenly ITC and 2-phenylethyl ITC. The concentration of the three ITCs changed similarly over time. In the midribs, ITC levels initially increased during storage (1 day for 20, 1 week at 4°C) but then generally decreased. ITCs dropped sharply at 20°C, but slowly at 4°C. During storage, leaf ITC levels

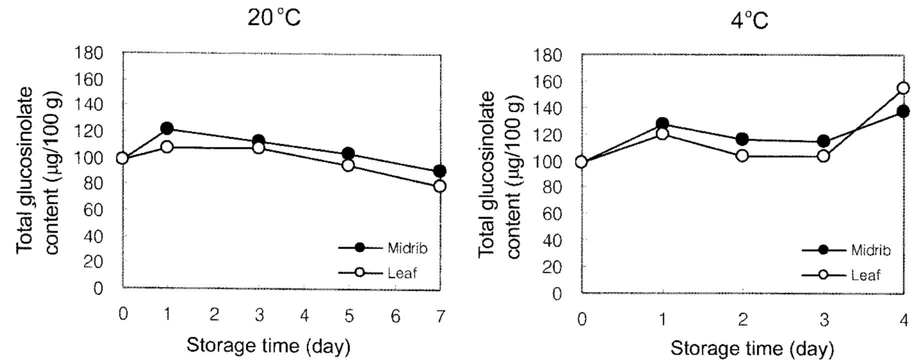

Fig. 4. Changes in total glucosinolate levels during *kimchi* storage.

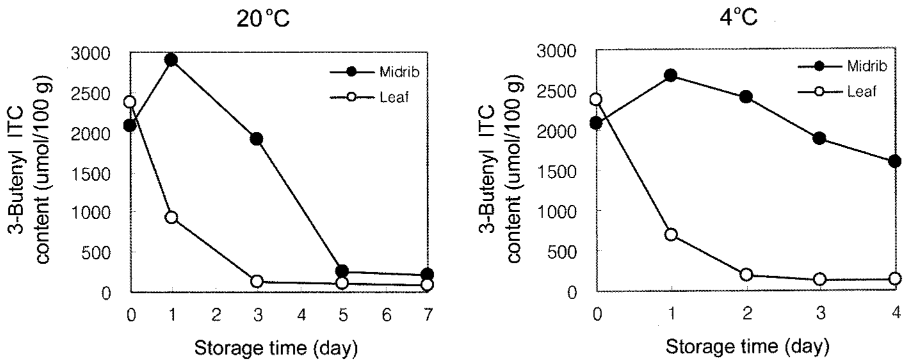

Fig. 5. Changes in 3-butenyl isothiocyanate levels during *kimchi* storage.

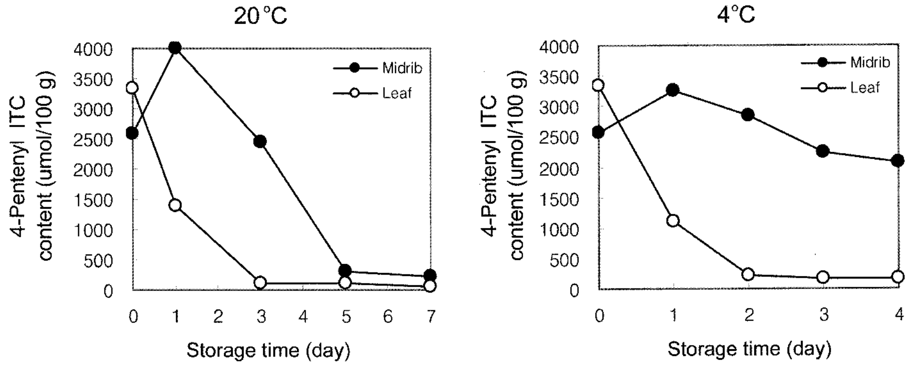

Fig. 6. Changes in 4-pentenyl isothiocyanate levels during *kimchi* storage.

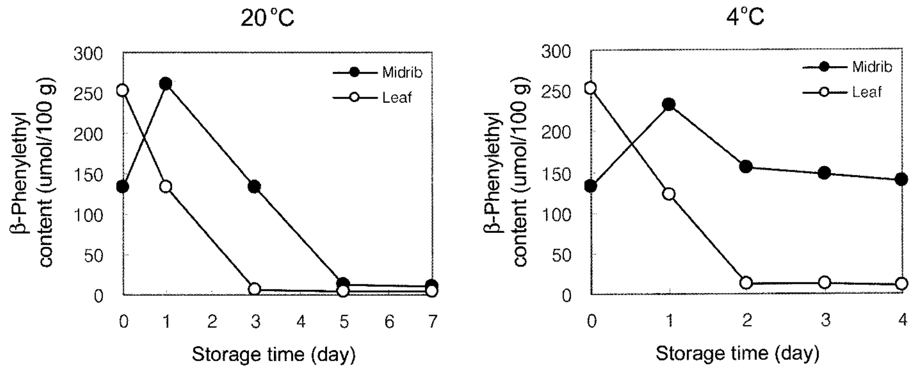

Fig. 7. Changes in β-phenylethyl isothiocyanate levels during *kimchi* storage.

fell markedly for 3 days at 20°C, and for 2 weeks in 20 at 4°C. The glucosinolates included a glucose and unstable aglycone moiety which subsequently rearranges to form different products, depending on the aglycone structure and the pH. Rearrangement of the aglycone usually results in the formation of an ITC at neutral pH, while at acidic pH the nitrile derivative is the dominant product (19). ITCs are also produced from myrosinase-catalyzed hydrolysis of glucosinolate in the extract (20). This reduction in ITC formation may have been caused by fading myrosinase activity during storage (21).

Based on these results, we conclude that storage-related pH changes reduce myrosinase activity sufficiently to lower the overall ITC content, although this overall tendency was initially opposed by an early increase of ITCs in the midrib that lasted for 1 day at 20°C, and 1 week at 4°C. In summary, the period of storage, temperature and cabbage section used for the preparation of *kimchi* are critical for the resulting glucosinolate and ITC levels.

Acknowledgments

This study was partly supported by 2006 research fund from DukSung Women's University, Korea.

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