**A THESIS FOR THE DEGREE OF MASTER OF SCIENCE**

# Deterioration Mechanism of Glucosinolate-Myrosinase System in Radish Roots during Cold Storage after Harvest

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**UNDER THE DIRECTION OF DR. EUN JIN LEE SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY**

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## ABSTRACT

The major isothiocyanates (ITCs) as a health benefit compounds in radish roots are raphasatin (RH) and sulforaphene (SFE). They are converted from precursor glucosinolates (GSLs), glucoraphasatin (GRH) and glucoraphenin (GRE), respectively, by activation of myrosinase. The overall process to form ITCs is called as glucosinolate-myrosinase (G-M) system. In this study, we investigated the G-M system in roots of six radish varieties to understand the changes of G-M system during

storage after harvest. Six varieties of radish roots were stored for 8 weeks at 0~1.5°C after modified atmosphere packaging. Five components consisting of G-M system, namely, ITCs concentration, GSLs concentration, myrosinase activity, ascorbic acid concentration, and myrosinase-related gene expression were measured respectively. The concentration of GRH and GRE, major GSLs of radish root, slightly decreased or maintained during entire storage days. However, the concentration of ITCs, especially SFE, and myrosinase activity were remarkably decreased until 8 weeks. Pearson correlation analysis using change rates of ITCs concentration, GSLs concentration, and myrosinase activity showed that a decrease of SFE concentration has a positive relationship ($r$ = 0.684, $P$ = 0.0017) to decrease of myrosinase activity during storage. Also, myrosinase activity related factors such as ascorbic acid concentration and myrosinase-binding protein gene expression were decreased during storage. Thus, a decrease of ITCs concentration that is the result of deterioration of G-M system might be caused by a decrease of myrosinase activity and myrosinase related factors. Our findings will provide an advanced view in order to maintain the quality of radish roots during storage, focusing on ITCs concentration, myrosinase activity, and myrosinase related factors but not GSLs concentration.

Keyword: cold storage, glucosinolate, isothiocyanate, myrosinase, *Raphanus sativus*

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# INTRODUCTION

Radish (*Raphanus sativus* L.), staple vegetable in many parts of the world, is a root vegetable of *Brassicaceae* family that has numerous varieties. Radish roots contain health beneficial compounds including sulfur compounds as glucosinolates (GSLs) (Gutiérrez and Perez, 2004). In Korea, harvested radish roots in fall are immediately transported to the market or commonly stored with modified atmosphere packaging (MAP) at 0~3°C for 1~4 months.

GSLs are nitrogen- and sulfur-containing secondary metabolites found in *Brassicaceae* family (Rosa et al., 1997) and do not have a direct biological activity (Musk et al., 1995). When plant tissue damaged by mechanical treatments (chewing, chopping or cutting) by insects or humans, myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), separated from GSLs into vacuoles of myrosin cell (Bones and Rossiter, 2006), hydrolyze GSLs to form several products like isothiocyanates (ITCs), nitriles, thiocyanates, and epithionitriles (Kissen et al., 2009; Winde and Wittstock, 2011). Degradation products of GSLs have a biological activity. The process to form the enzymatic products, especially ITCs, is called glucosinolate-myrosinase (G-M) system (Fig. 1).

ITCs are most functional phytochemicals among GSLs degradation products. In plant, ITCs are considered to play a role in the defense against herbivore (Beekwilder et al., 2008), also, have biological activity in human like antimicrobial

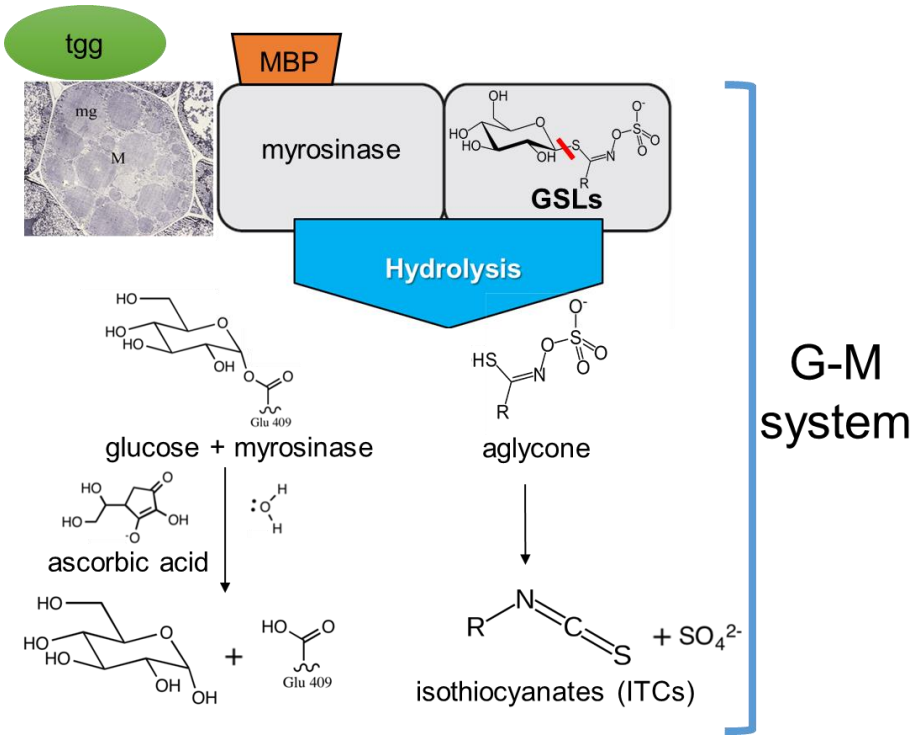


**Fig. 1.** Model of glucosinolate-myrosinase (G-M) system. tgg, thioglucoside glucohydrolase; MBP, myrosinase-binding protein, respectively.

(Esaki and Onozaki, 1982; Fahey et al., 2002), antioxidant (Barillari et al., 2006; Katzusaki et al., 2004; Takaya et al., 2003), and anticarcinogenic properties (Barillari et al., 2007; Hanlon et al., 2007). The major ITCs of radish roots, raphasatin (RH) and sulforaphene (SFE) (Scholl et al., 2011) are converted from precursors of ITCs, glucoraphasatin (GRH) and glucoraphenin (GRE), respectively, by activation of G-M system (Hanlon and Barnes, 2011).

ITCs commonly tend to decrease after harvest in radish roots because of deterioration of G-M system during storage (Lim et al., 2015). However, there is little information about what factors affect to deterioration of G-M system. The objective of this study is to discover the deterioration mechanism of G-M system of radish roots during storage. In six varieties of radish roots, the major ITCs RH and SFE contents were analyzed. Also, precursors of RH and SFE, GRH and GRE contents and myrosinase activity were analyzed.

In this study, we expect to elucidate the deterioration mechanism of G-M system causing decreases of ITCs in radish roots during storage. The result can be applied to postharvest technology to maintain G-M system, or to breeding of high nutritious radish cultivars. Furthermore, ultimate consumer will intake more healthy and nutritious radish roots.

# LITERATURE REVIEWS

## Radish roots

Radish, popular vegetable cultivated around the world, is a root vegetable of *Brassicaceae* family that has numerous varieties. Radish roots are good sources of vitamin C, folic acid, minerals, polyphenols, and GSLs (Hanlon and Barnes, 2011). Radish roots can be consumed in different forms for example processed or fresh to various products. Before radish roots are consumed, sometimes they are to be stored for 1~4 months.

For long-term storage, cold storage (Thompson, 2003) combined with MAP (Schreiner et al., 2003) is recommended. Beneficial MAP can be achieved within packages by wisely choosing the packaging material to help maintain appropriate oxygen and carbon dioxide levels (Sandhya, 2010).

## Glucosinolates

All GSLs have a core structure like a $\beta$-thioglucoside moiety, a sulfonated oxime aglycone, and a variable side chain derived from amino acids. To date, about 130 different side chains of GSLs have been reported. GSLs are classified by precursor amino acids and types of their side chains. GSLs derived from methionine, leucine,

or isoleucine are classified as aliphatic GSLs, derived from phenylalanine or tyrosine are classified as aromatic GSLs, and derived from tryptophan are classified as indole GSLs (Fahey et al., 2001). In radish roots, the major GSLs are GRH and GRE, derived from methionine. GRH is the predominant GSL accounting for about 74% of the total GSLs while GRE is the second most common GSL and accounts for less than 10% of the total GSLs in radish roots (Hanlon and Barnes, 2011).

There are many studies about the contents of GSLs during development stage or between plant organs. However, few studies about the contents of GSLs after harvest was conducted. Rodrigues and Rosa (1999) evaluated GSLs levels in the secondary inflorescences of fresh broccoli during storage at 4°C and 20°C. Inflorescences stored for 5 days showed a decrease in total GSLs of 4% and 64%, respectively. The glucoraphanin (GRA), major GSL in broccoli, content in florets declined by 82% after 5 days at 20°C, but declined only 31% at 4°C. Rangkadilok et al. (2002) reported approximately 50% decrease of GRA in broccoli heads in plastic bags after 7 days at 20°C, but no decrease was found after 7 days storage at 4°C.

## Myrosinase

Myrosinase, belonging to a family of glycoside hydrolases, is the only known enzyme that cleaves a thio-linked glucose found in nature. In presence of water, myrosinase cleaves off the glucose from GSLs (Holley and Jones, 1985). However, myrosinase and their substrate GSLs are stored in separate and different cell types

(Winde and Wittstock, 2011). While GSLs are stored in "S-cell" (Koroleva et al., 2000), myrosinase is stored as the tonoplast-like membranes surrounding myrosin grains within myrosin cells (Höglund et al., 1992). Myrosinase enzyme is encoded by thioglucoside glucohydrolase (TGG) gene family. TGG4 (Atlg47600) has been shown to encode functional myrosinase and seem to have root specific expression patterns (Andersson et al., 2009). In addition, myrosinase-binding protein (MBP) can interact with the myrosinase and then form myrosinase complex. MBP has been known to contribute to myrosinase activity in different *Brassica* species (Rask et al., 2000; Capella et al., 2001).

Ascorbic acid is a known cofactor of myrosinase, serving as a base catalyst in glucosinolate hydrolysis. For example, myrosinase isolated from white radish demonstrated an increase in activity from 2.06 µmol/min per mg of protein in the absence of ascorbic acid to 280 µmol/min per mg of protein in the presence of 500 µM ascorbic acid on the substrate, allyl glucosinolate (Shikita et al., 1999).

## Glucosinolate degradation products

The hydrolysis of GSLs by myrosinase can yield a variety of products, depending on various physiological conditions such as pH and the presence of certain cofactors. All known reactions have been observed to share the same initial steps. First, the *β*-thioglucoside linkage is cleaved by myrosinase, releasing D-glucose. The resulting aglycone undergoes a spontaneous Lossen-like rearrangement, releasing a

sulfate (Bones and Rossiter, 2006). The last step in G-M system is subject to the greatest variety depending on the physiological conditions under which the reaction takes place. At neutral pH, the primary product is ITCs. Under acidic conditions (pH < 5) and in the presence of ferrous ions, nitriles are formed (Galletti et al., 2001; Gil and MacLeod, 1980). When epithiospecifer proteins and ferrous ions are present, epithionitriles are formed (Lambrix et al., 2001).

In radish roots, the major ITCs are RH and SFE, degradation products of GRH and GRE, respectively. Although the contents of RH is higher than SFE, RH is less stable than its oxidized counterpart SFE in an aqueous environment. RH is degraded within 27.5 min after exposure to an aqueous environment, whereas SFE can remain stable for nearly 24 hours (Scholl et al., 2001).

# MATERIALS AND METHODS

## Plant materials

Six varieties of radish roots (*Raphanus sativus* L.), including 4 cultivars (Seoho, Cheonghwang, Junmuhumu, and Alpine) and 2 breeding lines (15FH352-1 and 15RA11-1) (Fig. 2), were harvested from an experimental field in Jeonju, Korea. After removing the leaves, radish roots were immediately transported to the laboratory.

## Storage of radish roots

Harvested radish roots were washed with 0.01% sodium hypochlorite for 3 min, washed twice with tap water, wiped with a paper towel to dry up, and pre-cooled at 2°C for 6 hour. Radish roots were covered with PE film during storage. In storage room, temperature was 0~1.5°C, and the relative humidity was maintained 85~90%. Three radish roots were sampled at 0 and 8 weeks and analyzed for ITCs contents, GSLs contents, and myrosinase activity. Middle area of roots was sliced to a 1-cm-thick disk and sliced into quarters. Sliced radish disks were stored in plastic bags at –95°C or freeze-dried.

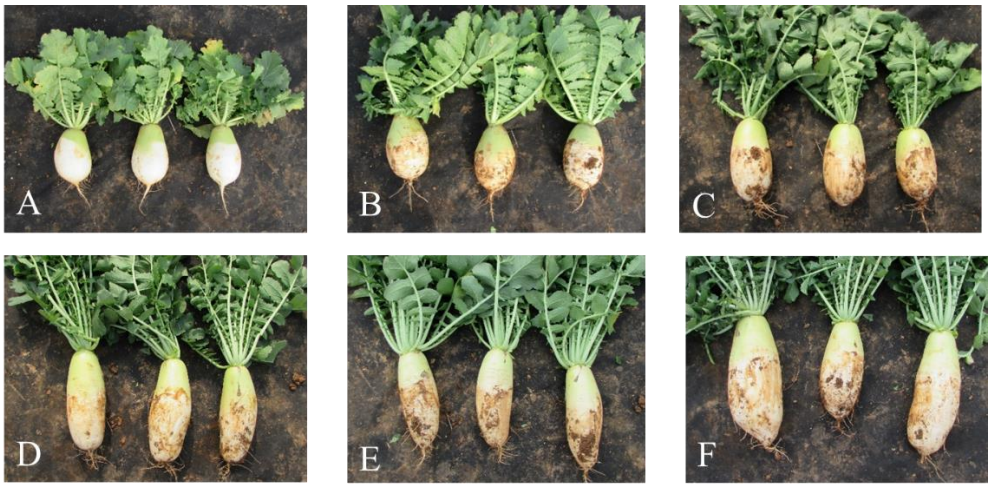


**Fig. 2.** Appearances of radish roots. A, 15RA11-1; B, 15FH352-1; C, Seoho; D, Cheonghwang; E, Junmuhumu; F, Alpine, respectively.

## Extraction of ITCs

The ITCs in the radish roots was extracted using liquid-liquid extraction methods (Kim et al., 2015) with slight modifications. Eight milliliters of methylene chloride (MC) and distilled water and 1 mL of 100 ppm benzyl ITC in MC as internal standard were added to 500 mg of lyophilized radish root powder. To hydrolyze endogenous GSLs to ITCs by myrosinase, a mixture was placed in a water bath at 37°C for 30 min. The hydrolyzed sample was added to 10 mL of MC and centrifuged at 3,000 rpm for 10 min. The MC layer was collected and filtered with filter paper containing anhydrous sodium sulfate to remove any water. The MC extract was evaporated to remove the MC under nitrogen evaporator at 30°C, re-dissolved in 1 mL of MC, and finally filtered with 0.45 μm syringe filter.

## Analysis of ITCs contents by GC-MS

The ITCs contents were analyzed by GC-MS analysis using the method described by Kim et al. (1997). The analysis was performed on a TRACE1310 GC system (Thermo, USA) with ISQ LT mass spectrophotometer (Thermo, USA) and DB-5 fused silica capillary column (0.25 × 30mm i.d., Agilent Technologies, USA). The oven temperature was set up to increase from 50 to 310°C at a rate of 5°C/min. The injector was split-less mode at 250°C. The flow rate of the helium was 1 mL/min. The range of mass scan was from 35 to 550 m/z.

## Extraction of the crude myrosinase

The crude myrosinase was extracted using the method (Andersson et al., 2009) with slight modifications. Fifty grams of radish root was homogenized with 80 mL of extraction buffer (10 mM potassium phosphate containing 3 mM DTT, 1 mM EDTA, and 5% glycerol) for 1 min. The mixture was immediately filtered with six layers of gauze and centrifuged at 6,700 rpm for 30 min at 4°C. The supernatant was saturated with ammonium sulfate to 55% and centrifuged at 6,700 rpm for 20 min at 4°C. The supernatant was saturated with ammonium sulfate to 80% and centrifuge at 6,700 rpm for 50 min at 4°C. The pellet was dissolve in 2 mL of desalting buffer (50 mM Tris-HCl, pH 8.6) and loaded to PD-10 column (GE healthcare, USA) equilibrated by 25 mL of desalting buffer. The desalted myrosinase was eluted with 3.5 mL of desalting buffer.

## Myrosinase activity assay

The method for myrosinase activity assay was investigated using a GO assay kit (Lim et al., 2015). One hundred microliters of crude myrosinase solution was added to 800 μL of 33 mM potassium phosphate buffer and 100 μL of 2 mM sinigrin. The mixture was incubated at 37°C for 30 min, placed in a heat-block at 95°C for 10 min to stop enzyme activity, and centrifuged at 13,500 rpm for 20 min. Fifty microliters of the supernatant was mixed with 100 μL of mixture regent in 96-well plate, incubated at 37°C for 30 min, and added to 100 μL of 12 N sulfuric acid to stop

enzyme activity. The absorbance of mixture was measured by microplate reader at 540 nm.

## Extraction of desulfo-GSLs

GSLs contents were determined by the desulfo-GSLs analysis method (Kliebenstein et al., 2001). Ten milliliters of boiled 70% methanol was added to 200 mg of lyophilized radish root powder and placed in a water bath at 90°C for 30 min, and centrifuged at 8,000 rpm for 30 min. The supernatant was evaporated under nitrogen evaporator at 40°C and re-dissolved in 1 mL of 30% methanol. The methanol extract was loaded to an ion-exchange column (5 mL of volume, Thermo scientific co., USA) filled with 100 mg of DEAE sephadex-A25 resin (GE healthcare, USA) and 1 mL of 2 M acetic acid. Next, the column was washed twice with 1 mL of buffer (20 mM sodium acetate, pH 5.0). Purified sulfatase (75 μL, 150 U/mL) was added onto the top of column and incubated at room temperature for 16 hour. Desulfo-GSLs were eluted 3 times with 0.5 mL of distilled water and freeze-dried. The lyophilized desulfo-GSLs was dissolved in 0.5 mL of distilled water and filtered with 0.45 μm syringe filter.

## Analysis of GSLs contents by HPLC

The desulfo-GSLs extracts were analyzed using YL 9100 HPLC system

(Younglin, Korea) with UV detector set at 229 nm and eclipse plus C18 column (4.6 × 250 mm, Agilent Technologies, USA). Initial condition of mobile phase was set up to 2% acetonitrile in water and maintained until 5 min. Flow rate was held constantly at 1.0 mL/min. A gradient mobile phase of water (A) and acetonitrile (B) separated the compounds by increasing B from 2% to 20% in 25 min and then switched to an additional linear gradient of 20% to 100% B up to 35 min.

## RNA extraction and qRT-PCR expression analysis

Total RNA was extracted from radish roots using HiGene total RNA Prep kit (Biofact, Korea). Radish roots were grounded in liquid nitrogen with a precooled pestle and mortar. Five hundred milligrams of ground radish roots was added to RB solution and centrifuged for 3 min at 14,000 rpm. The supernatant was washed twice with RNA washing solution and then RNA was extracted with DEPC water. cDNA synthesis was performed using amfiRivert platinum cDNA systhesis master mix kit (GenDEPOT, USA) for qRT-PCR. qRT-PCR was performed in a CMQE 500 (Cosmogenetech, Korea) in a final volume of 20 μL. Relative expressions were determined with normalization against the expression of radish *Actin* gene. The primers used for the qRT-PCR were listed in Table 1.

**Table 1.** Primers used for qRT-PCR.

| Gene | Forward primer sequence | Reverse primer sequence | Product size |
|---|---|---|---|
| TGG4 | TCGGAGACAGAGTCAAGTT | ATAAGGTTCGGTTCCAGAA | 136 |
| MBP | CCCTTCGCAGAAAGTAAAT | GAGGTATTGAGCAAATCAG | 141 |
| *Actin* | ATCATGGTGTCATGGTTGGG | GCCAGATCTTTTCCATATCA | 140 |

## Total ascorbic acid analysis

Total ascorbic acid analysis was conducted according to the methods described by Chebrolu et al. (2012) with some modifications. Five grams of frozen radish root was homogenized with *meta*-phosphoric acid extraction solution and centrifuged for 10 min at 8,000 rpm. Five hundred microliters of supernatant was added to 500 μL of 10 mM DTT solution. The mixture was analyzed using YL 9100 HPLC system (Younglin, Korea) with UV detector set at 252 nm and ZORBAX $NH_2$ column (4.6 × 250 mm, Agilent Technologies, USA). Mobile phase was 10 mM ammonium dihydrogen phosphate (pH 2.6) and flow rate was 1.0 mL/min.

## Statistical analysis

The data were statistically evaluated using SPSS statistical software. Experiments were conducted as randomized designs with three replicates. The means and standard deviations were calculated and the means were compared by the t-test at $P < 0.05$ (*) and $P < 0.01$ (**). Correlation analysis was conducted using change rates (8 weeks/0 week) of ITCs concentration, GSLs concentration and myrosinase activity. Partial least squares discriminant analysis (PLS-DA) was conducted using R statistical analysis program (version 3.0.1) to evaluate similarity among groups of multivariate data.

# RESULTS AND DISCUSSION

## ITCs concentration during cold storage

The GC-MS analysis of ITCs showed RH and SFE at 22.2 min and 29.1 min, respectively (Fig. 3). Changes of RH and SFE concentrations in radish roots during cold storage were shown in Figs. 4 and 5. The concentration of RH was slightly decreased in all radish roots during cold storage. Especially, the decrease in RH was noticeable in 'Seoho' radish root, decreasing from 0.076 to 0.025 mmol/g dry weight at 8 weeks. There were significant decreases in SFE concentrations in 'Seoho', 'Cheonghwang', 'Alpine', and '15RA11-1' radish roots during cold storage, by reducing 45%, 54%, 71%, and 30% of the SFE concentrations at 8 weeks, respectively.

A significant decrease in ITCs concentration was observed in the SFE concentration but not in the RH concentration. The reason of the result could be found in previous research for the stability of ITCs (Scholl et al., 2011). The half-life of the RH concentration is 27.5 min, whereas the SFE concentration maintains after 24 hours. This instability of RH could affect analysis accuracy of the RH concentration.

We confirmed the decreased concentration of SFE in radish roots during cold storage. These results agreed with those of Lim et al. (2015) who reported that there

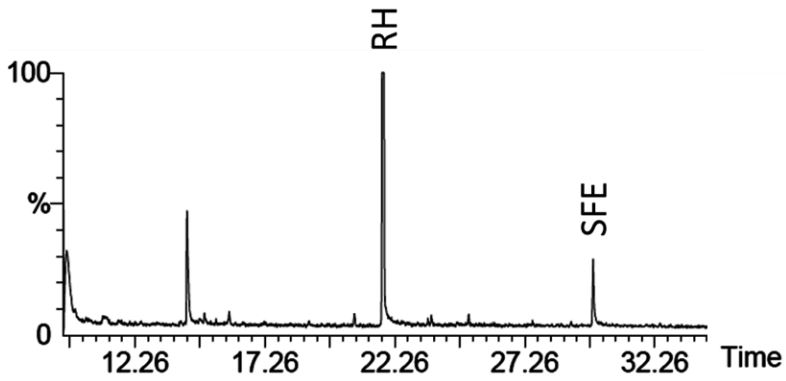


**Fig. 3.** GC-MS chromatogram of isothiocyanates in radish root. RH, raphasatin; SFE, sulforaphene.

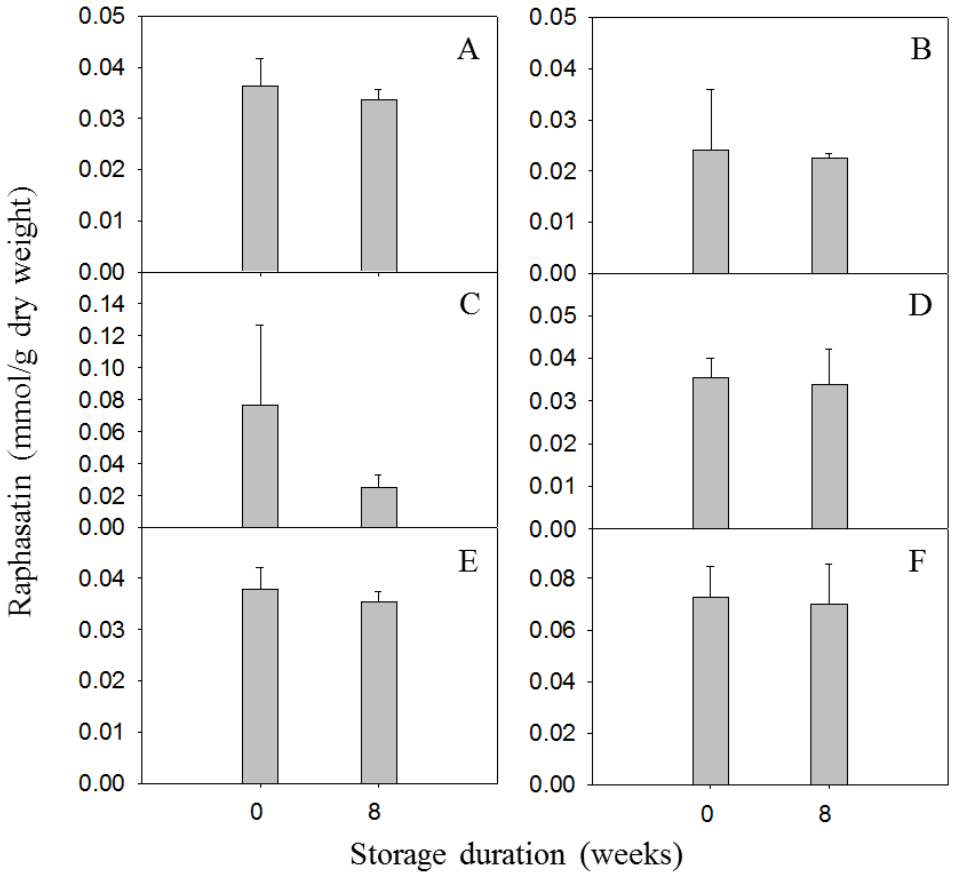


**Fig. 4.** Changes of raphasatin concentration in radish roots during cold storage. Vertical bars are means±SD (n=3). A, 15RA-11; B, 15FH352-1; C, Seoho; D, Cheonghwang; E, Junmuhumu; F, Alpine, respectively.

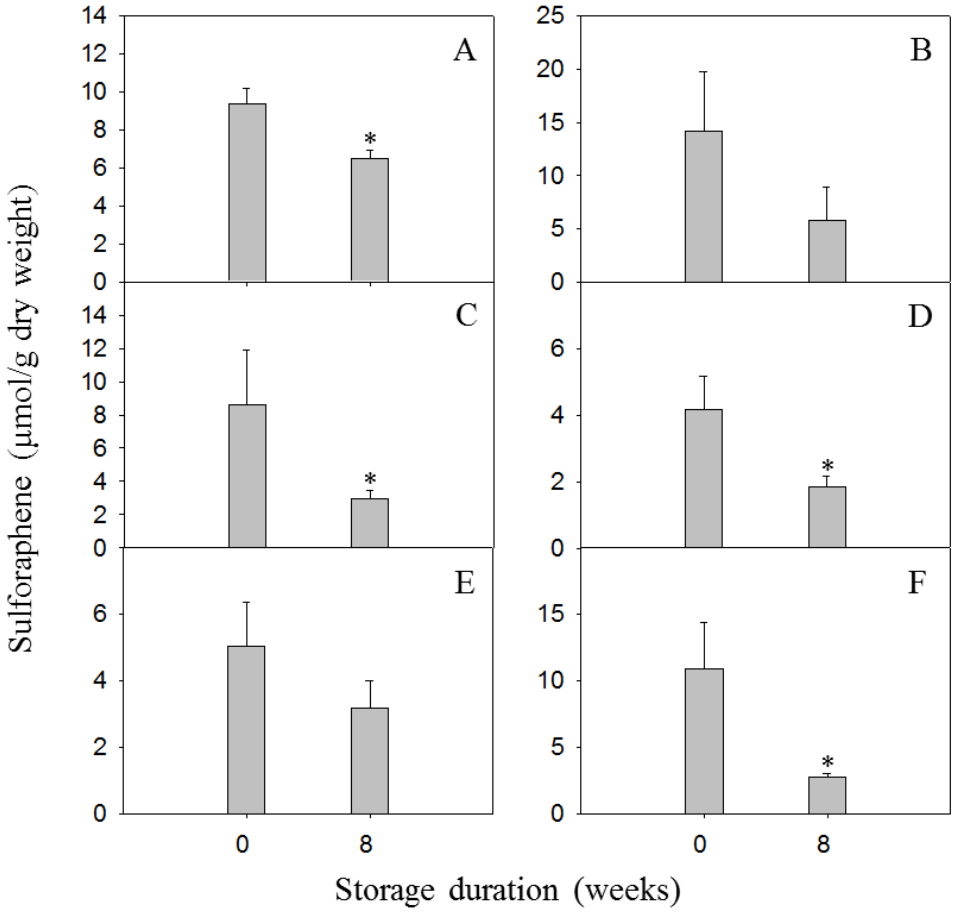


**Fig. 5.** Changes of sulforaphene concentration in radish roots during cold storage. Vertical bars are means±SD (n=3). A, 15RA11-1; B, 15FH352-1; C, Seoho; D, Cheonghwang; E, Junmuhumu; F, Alpine, respectively. *, significant at $P < 0.05$.

are an 39% reduction of the SFE concentrations in the ‘Chungwoon plus’ and 28% in the ‘Taebaek’ radish roots for 8 weeks cold storage, respectively.

## GSLs concentration during cold storage

In this study, the 8 kinds of GSLs in radish roots were detected using HLPC and LC-MS (Fig. 6). However, among 8 kinds of GSLs, only the GRH and GRE concentrations were quantified because GRH and GRE were precursors of RH and SFE, respectively. Changes of GRH and GRE concentrations in radish roots during cold storage were shown in Figs. 7 and 8. In ‘15RA11-1’, the GRH concentration was decreased until 55% of initial concentrations at harvest. In ‘Seoho’, the GRH concentration was increased to 133% of initial concentrations at harvest. Both the changes of GRH concentrations in ‘15RA11-1’ and ‘Seoho’ had a significance as $P<0.05$ during cold storage. The GRH concentration was increased in ‘Seoho’, ‘Cheonghwang’, and ‘Junmuhumu’ radish roots, however decreased in ‘Alpine’, ‘15FH352-1’, and ‘15RA11-1’ radish roots, respectively. However, most 4 cultivars except ‘15RA11-1’ and ‘Seoho’ were not significantly changed their GRH concentrations during storage. The GRE concentrations was increased in ‘Seoho’ radish root and decreased in other varieties, but all raw data had no significance. We confirmed that the GSLs concentration at 8 weeks after cold storage remained at harvest level or slightly decreased in 6 varieties. These results agreed with those of Rodrigues and Rosa (1999) who reported that the glucoraphanin, major GSL in

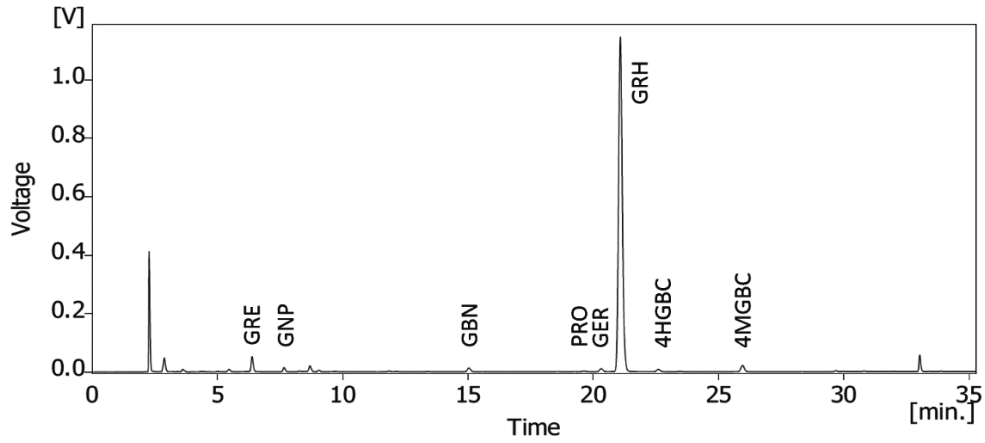


**Fig. 6.** HPLC chromatogram of glucosinolates in radish root. GRE, glucoraphenin; GNP, gluconapin; GBN, glucobrassicanapin; PRO, progoitrin; GER, glucoerucin; GRH, glucoraphasatin; 4HGBC, 4-hydroxyglucobrassicin; 4MGBC, 4-methoxyglucobrassicin, respectively.

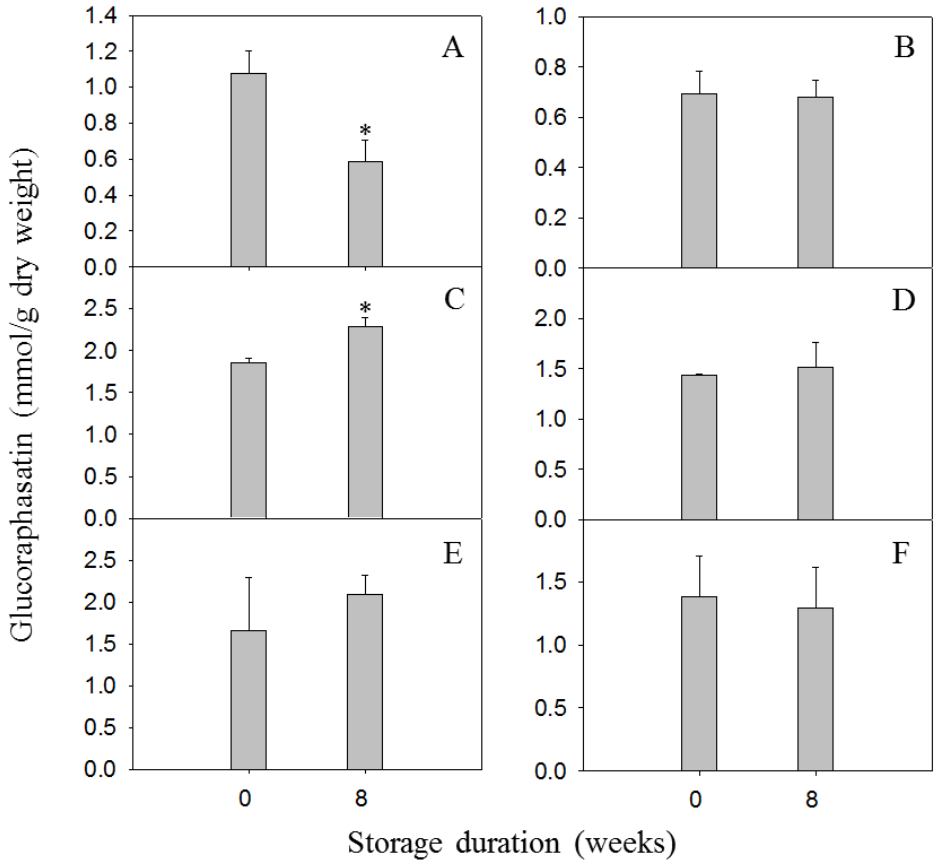


**Fig. 7.** Changes of glucoraphasatin concentration in radish roots during cold storage. Vertical bars are means±SD (n=3). A, 15RA11-1; B, 15FH352-1; C, Seoho; D, Cheonghwang; E, Junmuhumu; F, Alpine, respectively. *, significant at $P < 0.05$.

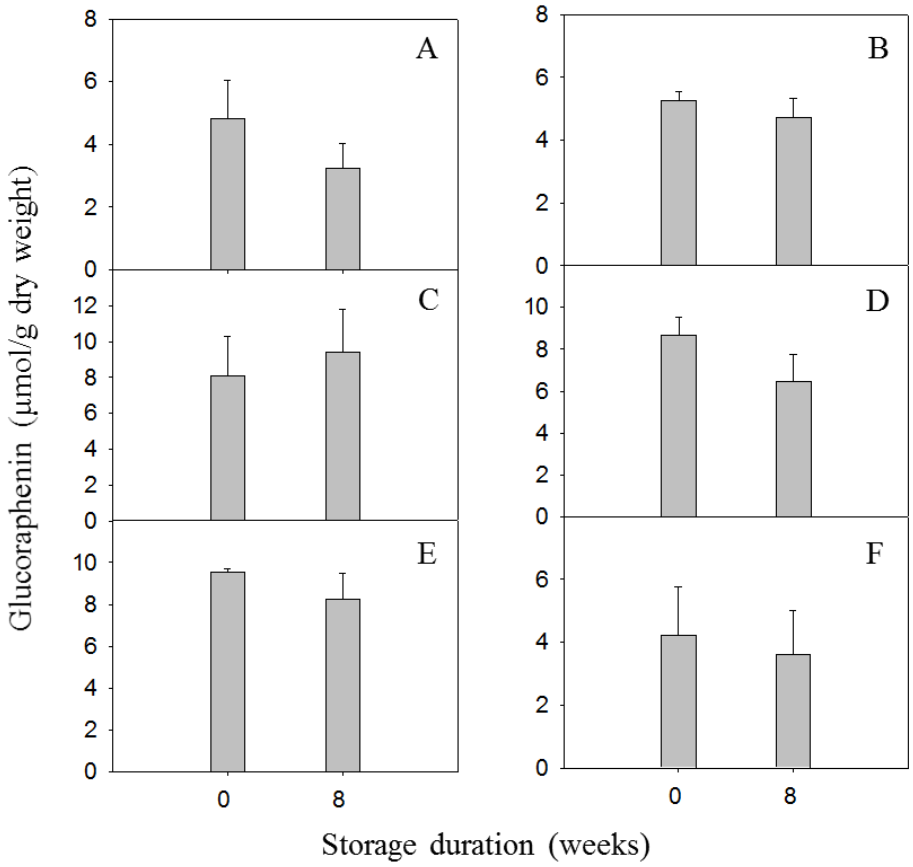


**Fig. 8.** Changes of glucoraphenin concentration in radish roots during cold storage. Vertical bars are means±SD (n=3). A, 15RA11-1; B, 15FH352-1; C, Seoho; D, Cheonghwang; E, Junmuhumu; F, Alpine, respectively.

broccoli, concentration decreases only 4% for 5 days at 4ºC and Rangkadilok et al. (2002) who reported that the GSLs concentration of broccoli stored in 4ºC with MAP for 25 days remains at harvest level.

## Myrosinase activity during storage

The activities of intact myrosinase in radish roots of 6 varieties were shown in Fig. 9. The activities of radish roots in 6 varieties were decreased during cold storage. Especially, 'Seoho', 'Cheonghwang', and '15FH352-1' radish roots showed significant decrease of the myrosinase activities, by reducing 10%, 11%, and 15% at 8 weeks, respectively ($P<0.05$). These results agreed with those of Lim et al. (2015) who reported that there are an 13% reduction of the myrosinase activity in the 'Chungwoon plus' and 15% in the 'Taebaek' radish roots for 8 weeks cold storage, respectively.

## Correlations among change rates of ITCs concentration, GSLs concentration, and myrosinase activity during storage

We conduct Pearson's correlation analysis and PLS-DA to confirm the reason of the significant decrease in the ITCs concentration of radish roots during storage. Pearson's correlations analysis was conducted by calculating change rate of ITCs

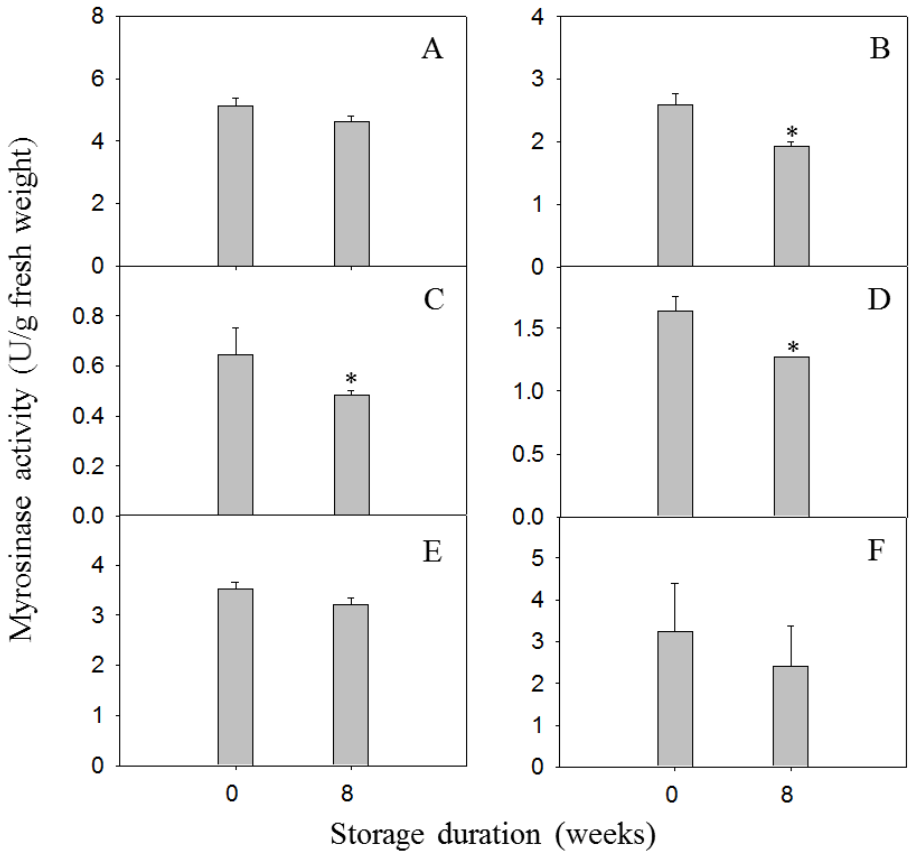


**Fig. 9.** Changes of myrosinase activity in radish roots during cold storage. Vertical bars are means±SE (n=3). A, 15RA11-1; B, 15FH352-1; C, Seoho; D, Cheonghwang; E, Junmuhumu; F, Alpine, respectively. U, nmol/min. *, significant at $P < 0.05$.

concentration, GSLs concentration, and myrosinase activity during storage. Fig. 10 showed that the change rates of RH concentration had a negative correlation with the change rates of GRH concentration ($r = –0.204^{ns}$) and had a positive correlation with the change rates of the myrosinase activity ($r = 0.174^{ns}$) with no significance. Fig. 11 explain that the change rates of SFE concentration had a weak negative correlation with the change rates of GRE concentration ($r = –0.130^{ns}$), but, had a definite positive correlation with the change rates of myrosinase activity ($r = 0.684^{**}$, $P < 0.01$), respectively.

The ITCs and GSLs concentration and the myrosinase activity in radish roots during cold storage were examined by PLS-DA (Fig. 12), with two principal components explaining 65.34% of the overall variances (46.52% and 18.82% for component 1 and component 2, respectively). In PLS-DA score plot, sample at 0 week located on right side and sample at 8 weeks located on left side. Also, we confirmed that the horizontal discrimination between each group for 0 week and 8 weeks was almost affected by the SFE concentration and the myrosinase activity through PLS-DA loading plot. These results finally mean that the SFE concentration and the myrosinase activity clearly decreased in radish roots during cold storage and the decrease of SFE concentration in radish roots during cold storage could be affected by the decrease of myrosinase activity more than the decrease of GSLs concentration.

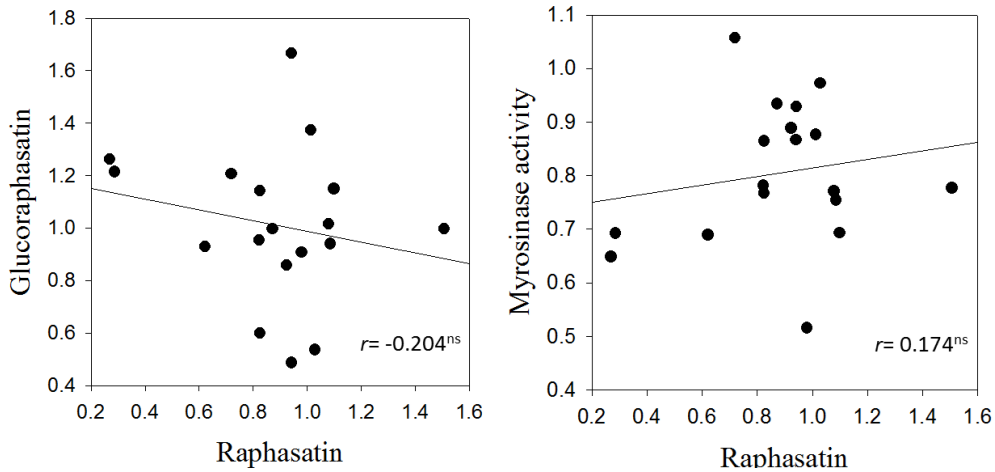


**Fig. 10.** Correlation analysis of change rates of raphasatin with change rates of glucoraphasatin and myrosinase activity in radish roots during cold storage. The correlation plot was based on linear regression analysis. *r*, Pearson correlation coefficient; ns, non-significant.

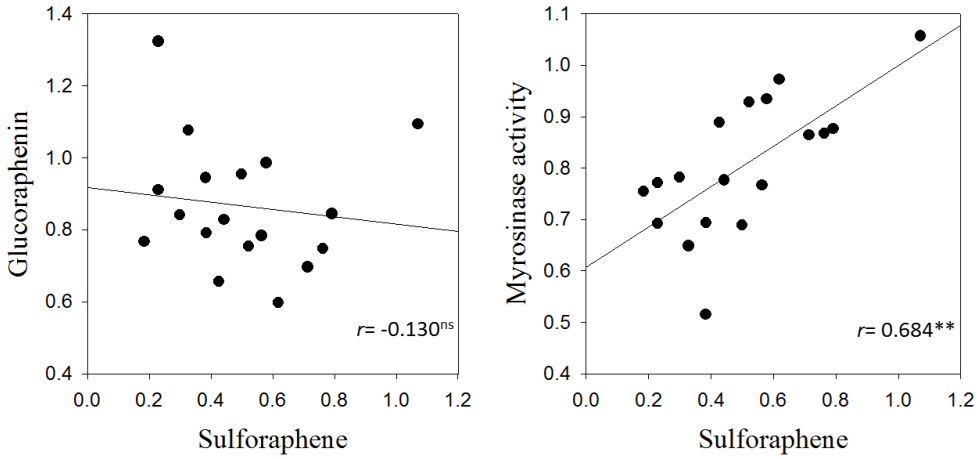


**Fig. 11.** Correlation analysis of change rates of sulforaphene with change rates of glucoraphenin and myrosinase activity in radish roots during cold storage after harvest. The correlation plot was based on linear regression analysis. *r*, Pearson correlation coefficient; ns, non-significant; **, significant at $P < 0.01$.

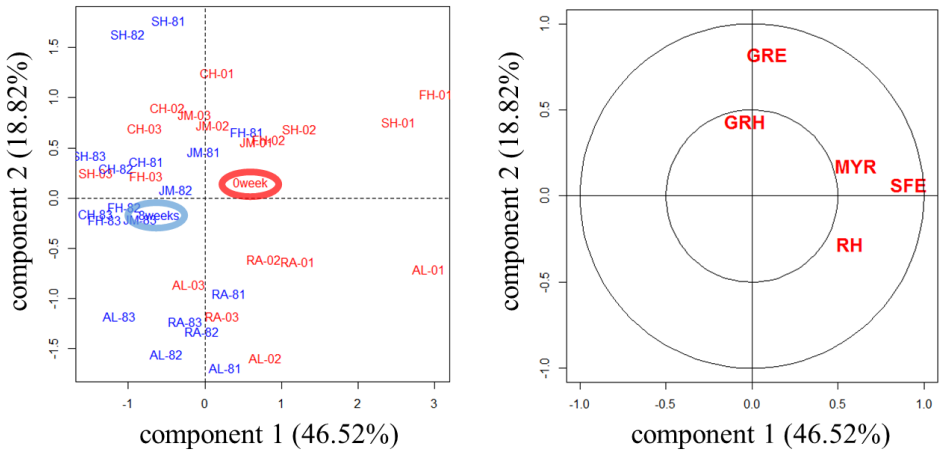


**Fig. 12.** PLS-DA score plot (left) and loading plot (right) of glucosinolats (GRH, glucoraphasatin; GRE, glucoraphenin), isothiocyanates (RH, raphasatin; SFE, sulforaphene), and myrosinase activity (MYR). SH, Seoho; FH, 15FH352-1; RA, 15RA11-1; CH, Cheonghwang; JM, Junmuhumu; AL, Alpine, respectively.

## Ascorbic acid concentration during storage

Total ascorbic acid concentration in radish roots were declined during cold storage (Fig. 13). These decrease had high significance in all 6 varieties as $P < 0.01$. The ‘Seoho’ cultivar had a biggest decrease in 6 varieties as 35% and others showed reduction of ascorbic acid concentration ranged from 10% to 20%.

According to Burmeister et al. (2000), myrosinase is strongly activated by ascorbic acid because ascorbic acid is a known cofactor of myrosinase, serving as a base catalyst in glucosinolate hydrolysis. Consequently, the decrease of myrosinase activity in radish roots during storage might be affected by the decrease of ascorbic acid concentration.

## Quantitative analysis of myrosinase related gene expression during storage

We conduct qRT-PCR to confirm relative expression of TGG and MBP gene related to myrosinase synthesis and activity, respectively. Changes of TGG and MBP relative gene expressions in radish roots during cold storage are shown in Figs. 14 and 15. In ‘Cheonghwang’ and ‘Alpine’ radish roots, the TGG gene expression was increased, however the gene expression was decreased in ‘15RA11-1’, ‘15FH352-1’, ‘Seoho’, and ‘Junmuhumu’ radish roots, but not showed significance. On the other hands, the MBP gene expression was decreased in radish roots of 6

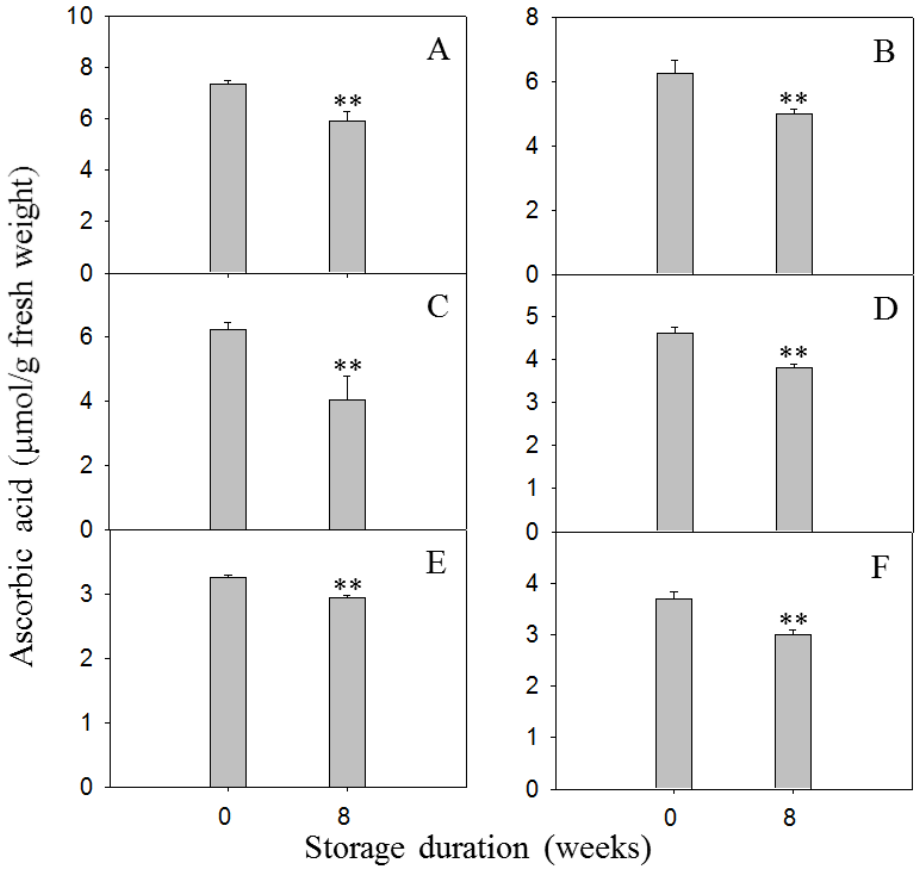


**Fig. 13.** Changes of ascorbic acid concentration in radish roots during cold storage. Vertical bars are means±SD (n=3). A, 15RA11-1; B, 15FH352-1; C, Seoho; D, Cheonghwang; E, Junmuhumu; F, Alpine, respectively. **, significant at $P < 0.01$.

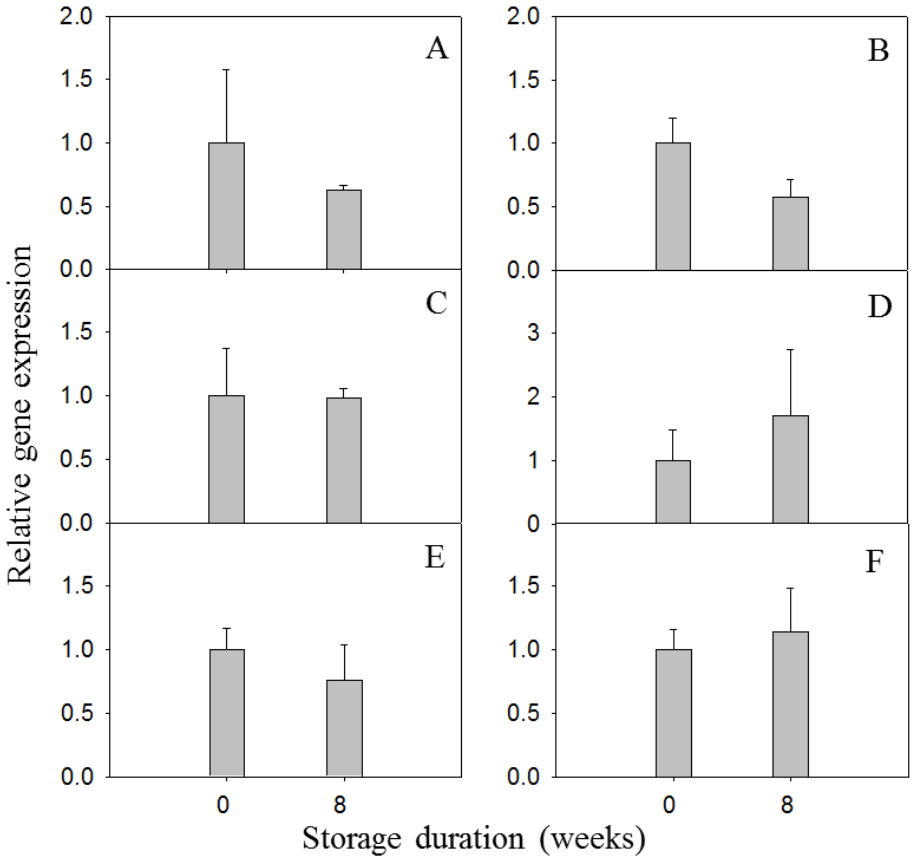


**Fig. 14.** TGG4 gene expressions in radish roots during cold storage. Vertical bars are means±SE (n=3). A, 15RA11-1; B, 15FH352-1; C, Seoho; D, Cheonghwang; E, Junmuhumu; F, Alpine, respectively.

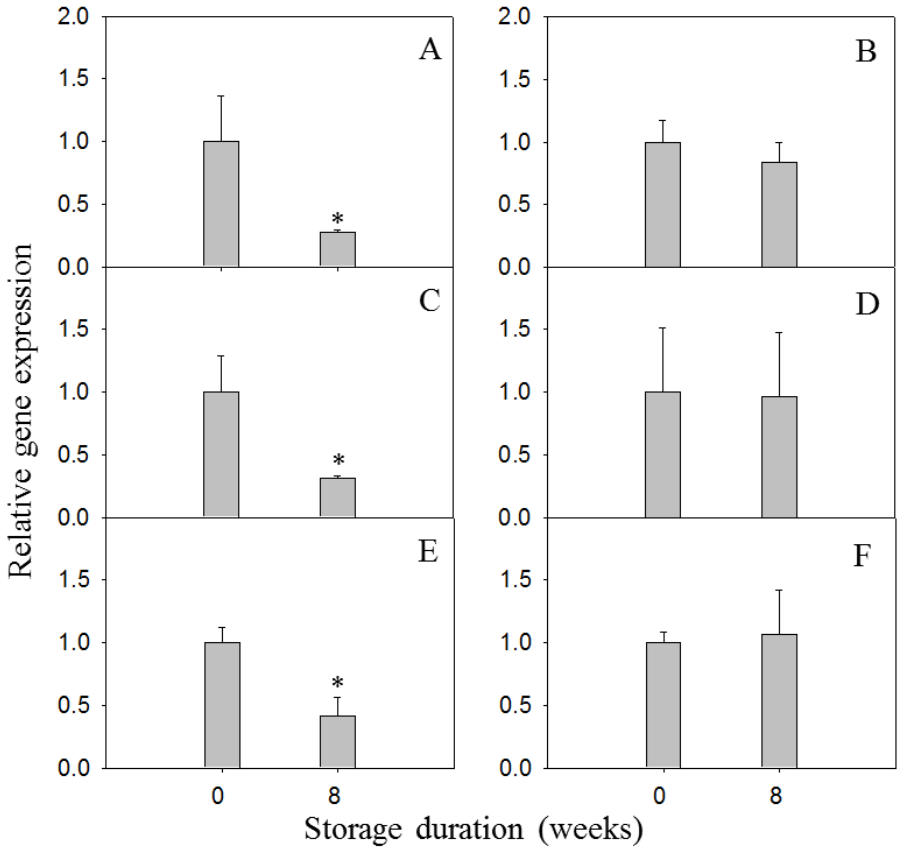


**Fig. 15.** MBP gene expressions in radish roots during cold storage. Vertical bars are means±SE (n=3). A, 15RA11-1; B, 15FH352-1; C, Seoho; D, Cheonghwang; E, Junmuhumu; F, Alpine, respectively. *, significant at $P < 0.05$.

varieties except ‘Alpine’ radish roots. Especially, ‘15RA11-1’, ‘Seoho’, and ‘Junmuhumu’ radish roots showed significant decreases of the MBP gene expression, by reducing 65%, 63%, and 61% at 8 weeks, respectively. As a result, we expect that the decrease of myrosinase activity was related with activation mechanisms through down regulation of MPB gene during storage, but not related with degradation of myrosinase synthesis such as TGG gene.

# CONCLUSION

Radish roots are a health beneficial vegetable for human, however, the huge decreases of bioactive compound as ITCs are occurred during cold storage. In this study, we demonstrated that the concentrations of ITCs including SFE and RH were reduced in radish roots of 6 varieties during cold storage. To understand the decrease mechanism of ITCs during cold storage, we firstly attempted the analysis research of changes in G-M system by cold storage. As a result, we confirmed that the change of GSLs as a precursor of ITCs was not affected to decrease of ITCs during storage by no difference of GSLs concentration after harvest. The myrosinase activity and myrosinase related factors such as ascorbic acid concentration and MBP gene expression were significantly decreased by storage period in radish roots. Especially, we defined that the decrease of ITCs in radish roots during storage had a positively correlation with the decrease of myrosinase activity through Pearson's correlation analysis and PLS-DA. Therefore, we proposed that the decrease of ITCs concentration in radish roots during cold storage was caused by the G-M system deterioration focused on reductions of myrosinase activation. In this study, the new insight of the G-M system deterioration in radish roots would be lead toward rationale postharvest biology and technology for enhanced quality including bioactive compound such as ITCs.

# REFERENCES

Andersson, D., D. Chakrabarty, S. Bejai, J. Zhang, L. Rask, and J. Meijer. 2009. Myrosinases from root and leaves of *Arabidopsis thaliana* have different catalytic properties. Phytochem. 70: 1345–1354.

Barillari, J., R. Cervellati, S. Costa, M. C. Guerra, E. Speroni, A. Utan, and R. Iori. 2006. Antioxidant and choleretic properties of *Raphanus sativus* L. sprout (Kaiware Daikon) extract. J. Agr. Food Chem. 54: 9773-9778.

Barillari, J., R. Iori, M. Broccoli, L. Pozzetti, D. Canistro, A. Sapone, and M. Paolini. 2007. Glucoraphasatin and glucoraphenin, a redox pair of glucosinolates of *Brassicaceae*, differently affect metabolizing enzymes in rats. J. Agr. Food Chem. 55: 5505-5511.

Beekwilder, J., W. Leeuwen, N. M. Dam, M. Bertossi, V. Grandi, L. Mizzi, M. Soloviev, L. Szabados, J. W. Molthoff, B. Schipper, H. Verbocht, R. C. H. Vos, P. Morandini, M. G. M. Aarts, and A. Bovy. 2008. The impact of the absence of aliphatic glucosinolates on insect herbivory in Arabidopsis. PLoS one 3: e2068.

Bones, A. M., and J. T. Rossiter. 2006. The enzymic and chmically induced decomposition of glucosinolates. Phytochem. 67: 1053-1067.

Burmeister, W. P., S. Cottaz, P. Rollin, A. Vasella, and B. Henrissat. 2000. High resolution X-ray crystallography shows that ascorbate is a cofactor for myrosinase

and substitutes for the function of the catalytic base. J. Biol. Chem. 275: 39385-39393.

Capella, A., M. Menossi, P. Arruda, and C. Benedetti. 2001. COI1 affects myrosinase activity and controls the expression of two flower-specific myrosinase-binding protein homologues in Arabidopsis. Planta 213: 691-699.

Chebrolu, K. K., G. K. Jayaprakasha, K. S. Yoo, J. L. Jifon, and B. S. Patil. 2012. An improved sample preparation method for quantification of ascorbic acid and dehydroascorbic acid by HPLC. LWT-Food Sci. Technol. 47: 443-449.

Esaki, H., and H. Onozaki. 1982. Antimicrobial action of pungent principles in radish root. J. Jpn. Soc. Nutr. Food Sci. 35: 207-211.

Fahey, J. W., X. Haristoy, P. M. Dolan, T. W. Kensle, I. Scholtus, K. K. Stephenosn, P. Talalay, and A. Lozniewski. 2002. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo [*a*] pyrene-induced stomach tumors. PNAS. 99: 7610-7615.

Fahey, J. W., A. T. Zalcmann, and P. Talalay. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochem. 56: 5-51.

Falk, A., J. Taipalensuu, B. Ek, M. Lenman, and L. Rask. 1995. Characterization of rapeseed myrosinase-binding protein. Planta 195: 387-395.

Galletti, S., R. Bernardi, O. Leoni, P. Rollin, and S. Palmieri. 2001. Preparation and biological activity of four epiprogoitrin myrosinase-derived products. J. Agric. Food

Chem. 49 :471–476.

Gil, V., and A. J. MacLeod. 1980. The effects of pH on glucosinolate degradation by a thioglucoside glucohydrolase preparation. Phytochem. 19: 2547–2551.

Gutiérrez, R. M. P., and R. L. Perez. 2004. *Raphanus sativus* (Radish): their chemistry and biology. The Sci. World J. 4: 811-837.

Hanlon, P. R., D. M. Webber, and D. M. Barnes. 2007. Aqueous extract from Spanish black radish (*Raphanus sativus* L. Var. *niger*) induces detoxification enzymes in the HepG2 human hepatoma cell line. J. Agr. Food Chem. 55: 6439-6446.

Hanlon, P. R., and D. M. Barnes. 2011. Phytochemical composition and biological activity of 8 varieties of radish (*Raphanus sativus* L.) sprouts and mature taproots. J. Food Sci. 76: C185-C192.

Höglund, A. S., M. Lenman, and L. Rask. 1992. Myrosinase is localized to the interior of myrosin grains and is not associated to the surrounding tonoplast membrane. Plant Sci. 85: 165-170.

Holley, R. A., and J. D. Jones. 1985. The role of myrosinase in the development of toxicity toward *Nematospora* in mustard seed. Can. J. Botany. 63: 521-526.

Katsuzaki, H., Y. Miyahara, M. Ota, K. Imai, and T. Komiya. 2004. Chemistry and antioxidative activity of hot water extract of Japanese radish (daikon). Biofactors 21: 211-214.

Kim, M. R., K. J. Lee, and H. Y. Kim. 1997. Effect of processing on the content of

sulforaphane of broccoli. Korean J. Soc. Food Sci. 13: 422–426.

Kim, J. W., M. B. Kim, and S. B. Lim. 2015. Formation and Stabilization of Raphasatin and Sulforaphene from Radish Roots by Endogenous Enzymolysis. Prev. Nutr. Food Sci. 20: 119.

Kissen, R., J. T. Rossiter, and A. M. Bones. 2009. The 'mustard oil bomb': not so easy to assemble?! Localization, expression and distribution of the components of the myrosinase enzyme systeme. Phytochem. Rev. 8: 69-86.

Kliebenstein, D. J., J. Kroymann, P. Brown, A. Figuth, D. Pedersen, J. Gershenzon, and T. Mitchell-Olds. 2001. Genetic control of natural variation in Arabidopsis glucosinolate accumulation. Plant Physiol. 126: 811-825.

Koroleva, O. A., A. Davies, R. Deeken, M. R. Thorpe, A. D. Tomos, and R. Hedrich. 2000. Identification of a new glucosinolate-rich cell type in Arabidopsis flower stalk. Plant Physiol. 124: 599-608.

Lambrix, V., M. Reichelt, T. Mitchell-Olds, D. J. Kliebenstein, and J. Gershenzon. 2001. The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. Plant Cell 13: 2793-2807.

Lim, S., E. J. Lee, and J. Kim. 2015. Decreased sulforaphene concentration and reduced myrosinase activity of radish root during cold storage. Postharvest Biol. Technol. 100: 219-225.

Musk, S. R. R., T. K. Smith, and I. T. Johnson. 1995. On the cytotoxicity and genotoxicity of allyl and phenethyl isothiocyanates and their parent glucosinolates sinigrin and gluconasturtiin, Mutat. Res. 348: 19-23.

Rangkadilok, N., B. Tomkins, M. E. Nicolas, R. R. Premier, R. N. Bennett, D. R. Eagling, and P. W. Taylor. 2002. The effect of post-harvest and packaging treatments on glucoraphanin concentration in broccoli (*Brassica oleracea* var. *italica*). J. Agr. Food Chem. 50: 7386-7391.

Rask, L., E. Andréasson, B. Ekbom, S. Eriksson, B. Pontoppidan, and J. Meijer. 2000. Myrosinase: gene family evolution and herbivore defense in *Brassicaceae*. Plant Mol. Biol. 42: 93-113.

Rodrigues, A. S., and E. A. S. Rosa. 1999. Effect of post-harvest treatments on the level of glucosinolates in broccoli. J. Sci. Food Agri. 79: 1028-1032.

Rosa, E. A. S., R. K. Heaney, G. R. Fenwick, and C. A. M. Portas. 1997. Glucosinolates in crop plants. Hort. Rev. 19: 99–215.

Sandhya. 2010. Modified atmosphere packaging of fresh produce: current status and future needs. LWT-Food Sci. Technol. 43: 381-392.

Scholl, C., B. D. Eshelman, D. M. Barnes, and P. R. Hanlon. 2011. Raphasatin is a more potent inducer of the detoxification enzymes than its degradation products. J. Food Sci. 76: 504–511.

Schreiner, M., S. Huyskens-Keil, A. Krumbein, H. Prono-Widayat, and P. Lüdders.

2003. Effect of film packaging and surface coating on primary and secondary plant compounds in fruit and vegetable products. J. Food Eng. 56: 237-240.

Shikita, M., J. W. Fahey, T. R. Golden, W. D. Holtzclaw, and P. Talalay. 1999. An unusual case of 'uncompetitive activation' by ascorbic acid: purification and kinetic properties of a myrosinase from *Raphanus sativus* seedlings. Biochem. J. 341: 725-732.

Takaya, Y., Y. Kondo, T. Furukawa, and M. Niwa. 2003. Antioxidant constituents of radish sprout (kaiware-daikon), *Raphanus sativus* L. J. Agr. Food Chem. 51: 8061-8066.

Thompson, A. K. 2003. Postharvest technology of fruits and vegetables. Harlow, Blackwell Science Ltd., Oxford.

Winde, I., and U. Wittstock. 2011. Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system. Phytochem. 72: 1566-1575.

# 초 록

무 뿌리의 대표적인 기능성 물질인 주요 isothiocyanates(ITCs)는 라파사틴과 설포라펜이다. 모든 ITCs는 전구물질인 글루코시놀레이트가 글루코시놀레이트-미로시네이즈(G-M) 시스템에 의해 분해되는 과정에서 생성된다. 따라서 본 연구에서는 저장 중 G-M 시스템의 변화를 이해하기 위하여 여섯 품종의 무 뿌리에서 G-M 시스템의 활성을 확인하였다. 여섯 품종의 무 뿌리는 modified atmosphere 포장 후에 8주간 1.5℃에 저장하면서 ITCs 함량, 글루코시놀레이트 함량, 미로시네이즈 활성, 아스코르브산 함량 및 미로시네이즈 관련 유전자 발현을 조사하였다. 저장 기간 중 글루코시놀레이트 함량은 크게 변화하지 않는 것을 확인하였으나, 설포라펜 함량과 미로시네이즈 활성은 8주간 감소하는 것을 확인하였다. ITCs 함량, 글루코시놀레이트 함량 및 미로시네이즈 활성 간의 Pearson 상관분석 결과 설포라펜의 감소는 미로시네이즈 활성의 감소와 정의 상관관계를 나타내는 것을 확인하였다($r$ = 0.684, $P$ = 0.0017). 또한, 미로시네이즈 활성과 관련된 요소인 아스코르브산 함량과 미로시네이즈 결합 단백질 유전자의 발현이 저장 기간 중 감소하는 것을 확인하였다. 이를 통하여 무 뿌리의 저장 기간 중 ITCs 함량의 감소는 전구물질인 글루코시놀레이트 함량의 감소에 의한 것이

아니라 글루코시놀레이트의 분해 효소인 미로시네이즈 활성이 감소하였기 때문에 나타난 결과임을 확인하였다.

주요어: 글루코시놀레이트, 미로시네이즈, isothiocyanate, *Raphanus sativus*

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