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碩士學位論文

# Optimization of Isothiocyanates Production from Glucosinolates in Radish Roots

濟州大學校 大學院

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指導敎授 任 尙 彬

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# Optimization of Isothiocyanates production from Glucosinolates in Radish Roots

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This thesis has been examined and approved.

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# Contents

# Abstract


The biologically active compounds, raphasatin and sulforaphene, are formed by the hydrolysis by an endogenous myrosinase in radishes. Raphasatin is very unstable during hydrolysis in aqueous media. This study determined the optimum hydrolysis conditions for maximizing the formation of raphasatin and sulforaphene by an endogenous myrosinase and for minimizing their degradations by the hydrolysis in radish roots. The reaction parameters, such as the reaction medium, reaction time, the type of mixing, and reaction temperature, were optimized. A stability test for raphasatin and sulforaphene was performed during storage of the hydrolyzed products at 25°C for 10 days. The formation and breakdown of raphasatin and sulforaphene in radish roots by endogenous enzyme was strongly influenced by the reaction medium, reaction time, and type of mixing. The production and stabilization of raphasatin in radishes was efficient in water and dichloromethane with shaking for 15 min at 25°C. For sulforaphene, water as a reaction medium without shaking for 10 min at 25°C was favorable condition. The maximum yields of raphasatin and sulforaphene were achieved in a concurrent hydrolysis reaction without shaking in water for 10 min and with shaking in dichloromethane for 15 min at 25°C. Under these conditions, the yields of raphasatin and sulforaphene were maximized at 12.89 and 1.93 μmol/g of dry radish, respectively. The stabilities of raphasatin and sulforaphene in the hydrolyzed products were 56.4 and 86.5% after 10 days of storage in water and dichloromethane at 25°C.

# 1. Introduction

Radishes (*Raphanus sativus L.*) belong to the family Brassicaceae, and are cultivated and consumed throughout the world. Radishes are a good source for vitamin C, dietary fibers, minerals, polysaccharides, gibberellins, alkaloids and nitrogenous compounds, as well as a large variety of phytochemicals, namely glucosinolates and phenols (Gutiérrez & Perez, 2004). The primary glucosinolates in radishes are thio-functionalized glucosinolates (GLs), glucoraphasatin (4-methyl thio-3-butenyl GL) and glucoraphenin (4-methyl sulfinyl-3-butenyl GL). Glucoraphasatin is the predominant GL, accounting for about 80% of the total, while glucoraphenin is the second most common GL and accounts for less than 10% of the total GLs in mature radishes (Hanlon & Barnes, 2011). In their original forms, these compounds do not perform any direct biological activities, but their derivatives i.e., isothiocyanates (ITCs), raphasatin (4-methyl thio-3-butenyl ITC) and sulforaphene (4-methyl sulfinyl-3-butenyl ITC), have potential health benefits.

Raphasatin is an antioxidant (Papi et al., 2008 Yuan, G., Wang, X., Guo, R., & Wang, Q., 2010) and a potent inducer of phase II enzymes in precision-cut rat liver slices (Abdull-Razis, De Nicola, Pagnotta, Iori, & Ioannides, 2012), is cytotoxic to multiple cancer cell lines (Hanlon, Webber, & Barnes, 2007 Barillari et al., 2008 Yamasaki et al., 2009 Beevi, Mangamoori, Subathra, & Edula, 2010), and has antimutagenic properties (Nakamura et al., 2001). Sulforaphene has antimicrobial, antiviral, and antioxidant properties (Nakamura et al., 2001; Ippoushi, Takeuchi, Ito, Horie, & Azuma, 2007; Yuan et al., 2010). Sulforaphene is 1.3-1.5-fold more active than sulforaphane with regard to *in vitro* antimutagenic activities in *salmonella typhimurium* strain in the presence of Aroclor 1254-induced rat liver S9 (Shishu & Kaur, 2009). Sulforaphene has greater cancer preventive properties (IC50 = 10.67 μg/mL) against the human colon cancer (HCT116) cell line than the chemotherapeutic drug, mitomycin C (IC50 = 19.12 μg/mL), via both cytotoxicity and the induction of

apoptosis (Pocasap, Weerapreeyakul, & Barusrux, 2013).

Radishes are generally utilized as raw vegetables and as components of salads. They may be more beneficial than other cruciferous vegetables, which are consumed after cooking, because heating inactivates myrosinase that is essential for producing active ITCs from their GL precursors (Shishu & Kaur, 2009). Glucoraphasatin and glucoraphenin in raw radish roots are converted to raphasatin and sulforaphene in water by endogenous myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) when plant tissues and cells are damaged. The capability of myrosinase in the degradation of GLs could be influenced by a number of processing factors, such as reaction temperature, reaction time, solid-liquid ratio, pH, reaction medium, type of mixing, vitamin C levels, etc. (Shen, Su, Wang, Du, & Wang, 2010). The stability of the hydrolyzed products, which are critical for their development as functional agents or as dietary foods, is particularly affected by the reaction medium.

Sulforaphene is slightly soluble in water and thus is produced and stabilized during the hydrolysis of radishes in an aqueous medium. However, raphasatin is hydrophobic, highly unstable in an aqueous environment, so generates and spontaneously converts to less active compounds during the hydrolysis of radishes in water (Li, Lee, Lee, Auh, Kim, & Yoon, 2010 Montaut, Barillari, Iori, & Rollin, 2010; Scholl, Eshelman, Barnes, and Hanlon, 2011). Therefore, there is a need to optimize a processing method to maximize the formation and minimize the degradation of raphasatin and sulforaphene by the endogenous enzymolysis of radishes.

Shen et al. (2010) investigated the conversion of glucoraphanin to sulforaphane using an endogenous myrosinase in broccoli seed powders under various enzymolysis conditions, such as reaction temperature, reaction time, solid-liquid ratio, pH, and the addition of vitamin C. However, a systematic study of endogenous enzymatic hydrolysis with radishes under various enzymolysis conditions has not been reported yet. Most previous studies have been performed on the hydrolysis of radishes in water or in 0.1 M phosphate buffer (pH 6.5) for a certain length of time, and the

resulting mixture was then centrifuged, with the addition of ethanol or dichloromethane for recovering the hydrolyzed products (Hanlon, Robbins, Hammon, & Barnes, 2009; Hanlon & Barnes, 2011 Scholl et al., 2011 Abdull-Razis et al., 2012 Pocasap et al., 2013 Li, J., Xie, B., Yan, S., Li, H., Wang Q., 2015). The formation and degradation of the hydrolyzed products in aqueous media were not considered during the hydrolysis and centrifugation processes. Therefore, the reported yields of ITCs from GLs could be inconsistent.

The objective of this study was to optimize the hydrolysis conditions to increase yields by minimizing the degradation of raphasatin and sulforaphene from glucoraphasatin and glucoraphenin by an endogenous myrosinase in the radish roots.

## 2. Materials and methods

### 2.1. Materials

Radish roots (Raphanus sativus L.) were obtained from a local store in Jeju, Jeju province, Korea. They were washed, sliced, and freeze dried. Radish roots powders were stored in a cooler at 4°C until needed. Sulforaphene, erucin, benzyl ITC (BITC) standards were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Sinigrin (2-propenyl GSL) was obtained from the Tokyo Kasei Kogyo Company (Tokyo, Japan). Diethylaminoethyl (DEAE)-sephadex A-25 was supplied by Amersham Biosciences (Uppsala, Sweden). Aryl sulfatase (Type H-1, EC 3.1.6.1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Wako Pure Chemical Industries (Osaka, Japan). HPLC-grade dichloromethane was purchased from Daejung Chemical Co., Ltd (Seoul, Korea). HPLC-grade methanol was purchased from Huadong Chemical Reagents Ltd. (Hangzhou, China).

### 2.2. Analyses of glucosinolates in radish roots

Glucosinolates in radish roots were measured based on the procedure used by Kim and Ishii (2007). Crude GLs from freeze-dried radish powders (100 mg) were extracted using 1.5 mL of boiling 70% (v/v) aqueous methanol in a water bath at 70°C for 5 min. After centrifugation (4°C, 12,000 × g, 10 min), the supernatant was transferred to a test tube, and the residue was further re-extracted twice. The crude GL extracts were loaded onto a pre-equilibrated column (DEAE-Sephadex A-25). After that the column was washed twice with 1 mL ultra pure water to remove neutral and positive ions, aryl sulfatase (E.C.3.1.6.1) (75 µL) was loaded onto the column. After the desulfation reaction proceeded overnight (16 h) at room

temperature, the desulfated GLs were eluted three times with 0.5 mL distilled water. The eluates were filtered through a 0.45 µm Teflon PTFE syringe filter (Millipore, Billerica, MA, USA) and analyzed by HPLC.

Glucosinolates were analyzed using a 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with an Inertsil ODS-3 column (150 × 3.0 mm i.d., particle size 3 mm, GL Science, Tokyo, Japan). A flow rate of 0.4 mL/min, a column temperature of 40°C, and a wavelength of 227 nm were used. The solvents were (A) ultra-pure water and (B) acetonitrile, and the program used was 0-2 min solvent B 0%, 7 min solvent B 10%, 16-19 min solvent B 31%, 21 min solvent B 0%, and then kept constant at solvent B 0% for 6 min.

*2.3. Analyses of isothiocyanates in radish roots*

The intrinsic ITC content of the radish roots before hydrolysis was measured. Ten milliliters of dichloromethane were added to 0.5 g of freeze-dried radish roots powder in a 50 mL centrifuge tube. The tube was left undisturbed at 25°C for 30 min, and then centrifuged at 7,000 × g and 4°C for 5 min, and the dichloromethane was collected and the residual liquid was extracted by dichloromethane until the third time using the same process, the combined extracts were filtered through a No. 5A filter paper (Advantec, Tokyo, Japan) with 2 g of anhydrous sodium sulfate. The filtrate was evaporated to remove the solvent using a rotary vacuum evaporator (Rotavapor R-124, BUCHI Labortechnik AG, Flawil, Switzerland) at room temperature. The dried residue was dissolved in 2 mL of dichloromethane and filtered using a 0.50 µm syringe filter (Advantec, Tokyo, Japan.). A 200 µL aliquot of a 75 µg/mL solution of BITC (internal standard) was added to 400 µL of this solution, and the sample was then analyzed for ITCs by gas chromatography / mass spectrometry (GC/MS).

Raphasatin and sulforaphene were analyzed using an Agilent 6890N GC / 5973 MSD (Agilent Technologies, Santa Clara, CA, USA). A HP-5 MS capillary column

(30 m × 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA) was used for separation. Helium was used as the carrier gas at a constant flow of 0.8 mL/min. The column temperature was maintained at 150°C for 8 min, and then programmed at 25°C/min to 250°C, where it remained for 2 min. The GC inlet temperature was 200°C and the transfer line temperature was 280°C. The injection volume was 2 µL, with a split ratio of 1:20. Mass spectra were obtained by electron-impact ionization over the mass range 50-500 amu at a scan rate of 2 scans/sec. Typical GC/MS chromatograms of BITC, erucin, and sulforaphene standards and the radish roots extracts are shown in Fig. 1.

The concentration of raphasatin was calculated using erucin as a standard, which is similar in structure and commercially available (Scholl et al., 2011). The concentration of sulforaphene was calculated using a sulforaphene standard. Standard solutions were prepared with dichloromethane for a set of erucin solutions at concentrations of 150, 175, 200, 225, and 250 µg/mL and a set of sulforaphene solutions with concentrations of 40, 50, 60, 70, and 80 µg/mL. A 100 µL aliquot of a 75 µg/mL solution of BITC (internal standard) was added to 200 µL of each standard solution, and the mixed standard solutions were then analyzed by GC/MS. The ratios of the peak area of erucin or sulforaphene to BITC for each solution were recorded and plotted against the concentrations to form the standard curves.
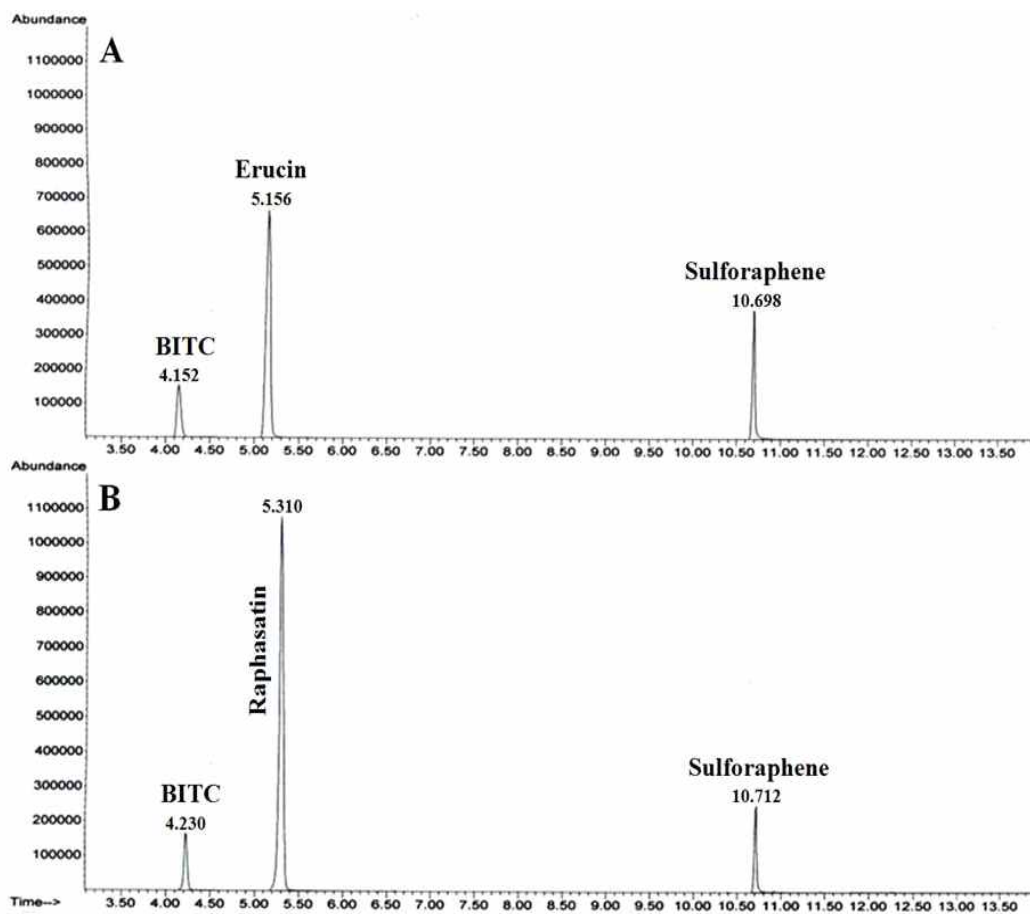
**Fig. 1.** Chromatograms of standards (A) and the radish roots extract (B).

*2.4. Endogenous enzymatic hydrolysis of radish roots under various conditions*

Eight milliliters of distilled water and/or dichloromethane were added gently to 0.5 g of freeze-dried radish roots powder in a 50 mL centrifuge tube. The endogenous enzymatic hydrolysis was performed at a controlled temperature for a certain length of time with and without shaking. The enzymatic reaction was stopped by the addition of 1.8 mL of 6 M HCl for 15 min. The resulting mixture was added to 10 mL of dichloromethane and centrifuged at 7,000 × g at 4°C for 5 min, after that the dichloromethane layer was collected. The rest of the experimental method was as described in Section 2.3.

The endogenous enzymatic hydrolysis was conducted under various controlled conditions, including different reaction media (distilled water (hydrophilic) and dichloromethane (hydrophobic)), reaction times (5, 10, 15, 20, and 25 min), type of mixing (with and without shaking), and reaction temperatures (10, 25, and 37°C). A stability test for the raphasatin and sulforaphene produced by enzymatic hydrolysis was also performed during storage of the hydrolysis products at 25°C for 2, 4, 6, 8, and 10 days.

*2.5. Statistical analyses*

Three replicate experiments were conducted and the data presented here represent the average of three experiments ± standard deviation. Statistical analyses were performed using the SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA). Significant differences ($p < 0.05$) among the treatment means were determined by Duncan's test.

# 3. Results and discussion

## 3.1. Glucosinolates and intrinsic isothiocyanates concentrations in radish roots

The glucosinolates concentration in freeze-dried radish roots was (in Table 1). Glucoraphasatin was the most abundant (13.53 µmol/g, 85.6%) of all GLs (15.79 µmol/g) in freeze-dried radish roots. The minor GLs detected were 4-methoxyglucobrassicin (0.47 µmol/g, 2.9%), glucoalyssin (0.42 µmol/g, 2.6%), glucobrassicanapin (0.41 µmol/g, 2.5%), glucoerucin (0.24 µmol/g, 1.5%), and glucobrassicin (0.23 µmol/g, 1.4%). Glucoraphenin was not detected because it was present at very low concentrations that were below the detection limit.

De Nicola et al. (2013) detected four GLs in the two *R. sativus* varieties: two aliphatic (glucoraphasatin and glucoraphenin), representing 96% of the total GLs, and two indolic (4-hydroxyglucobrassicin and 4-methoxyglucobrassicin), accounting for the remaining 4%. Hanlon and Barnes (2011) also found that in eight radish varieties of both sprouts and mature taproots, glucoraphasatin was the predominant GL. In sprouts, glucoraphenin was the second most common GL in all varieties; however, it accounted for less than 10% of the total GLs in all of the mature taproots.

The intrinsic ITC contents in freeze-dried radish roots before endogenous enzymatic hydrolysis in water were also measured with GC/MS by extracting freeze-dried radishes three times using dichloromethane. Only a small amount of raphasatin was detected (1.97 ± 0.01 µmol/g of dry radish), while sulforaphene was not detected. Both raphasatin and sulforaphene as health functional compounds should be hydrolyzed by the endogenous enzyme to maximize the yield.

**Table 1.** Glucosinolates content of radish roots.

| Retention time (min) | Glucosinolates | Content (μmol/g dry radish roots) |
|---|---|---|
| 9.18 | Progoitrin | 0.19 ± 0.01 |
| 11.06 | Glucoalyssin | 0.42 ± 0.05 |
| 13.61 | Glucocochlearin | 0.20 ± 0.05 |
| 15.23 | Glucobrassicanapin | 0.41 ± 0.02 |
| 15.50 | Glucoerucin | 0.24 ± 0.00 |
| 15.85 | Glucoraphasatin | 13.53 ± 0.81 |
| 16.19 | Glucobrassicin | 0.23 ± 0.03 |
| 17.08 | 4-Methoxyglucobrassicin | 0.47 ± 0.02 |
| 19.23 | Neoglucobrassicin | 0.09 ± 0.06 |
| | Total | 15.79 ± 0.89 |

*3.2. Conditions for stopping the enzyme reaction after hydrolysis*

To measure the effects of the hydrolysis reaction parameters on the formation of raphasatin and sulforaphene in radish roots, the enzyme reaction should be stopped after hydrolysis under certain enzymolysis conditions. The enzyme reaction will otherwise continue to occur during the following extraction and centrifugation processes for the recovery of the transformed raphasatin and sulforaphene after hydrolysis.

To determine the quantity of 6 M HCl required to stop the enzyme reaction, different quantities (0.2-3.4 mL) of 6 M HCl were added after hydrolysis, and the mixture was left for 15 min before the transformed ITCs were measured (Fig. 2). The raphasatin yield gradually decreased as the quantity of 6 M HCl was increased, before a constant value of about 2.52 μmol/g of dry radish was reached after the addition of 1.8 mL 6 M HCl. The yield of sulforaphene was constant at about 1.33 μmol/g of dry radish after 0.2 mL of 6 M HCl was added. This indicates that the glucoraphenin, which was present in small amounts in radishes, was almost completely transformed into sulforaphene within 5 min of hydrolysis, while glucoraphasatin, which was abundant in radishes, was continuously produced during the following extraction and centrifugation processes for the recovery of the transformed raphasatin after hydrolysis, until the endogenous myrosinase in radishes was inactivated with the addition of 1.8 mL of 6 M HCl.

The reaction time required to stop the enzyme reaction with 1.8 mL of 6 M HCl was also investigated (Fig. 3). The yield of raphasatin gradually decreased as the reaction time was increased, and then reduced to a constant value after 15 min, while the yield of sulforaphene remained unchanged regardless of the reaction time. Therefore, endogenous myrosinase in radishes was inactivated by the addition of 1.8 mL of 6 M HCl for 15 min, which was then applied in the following experiment.
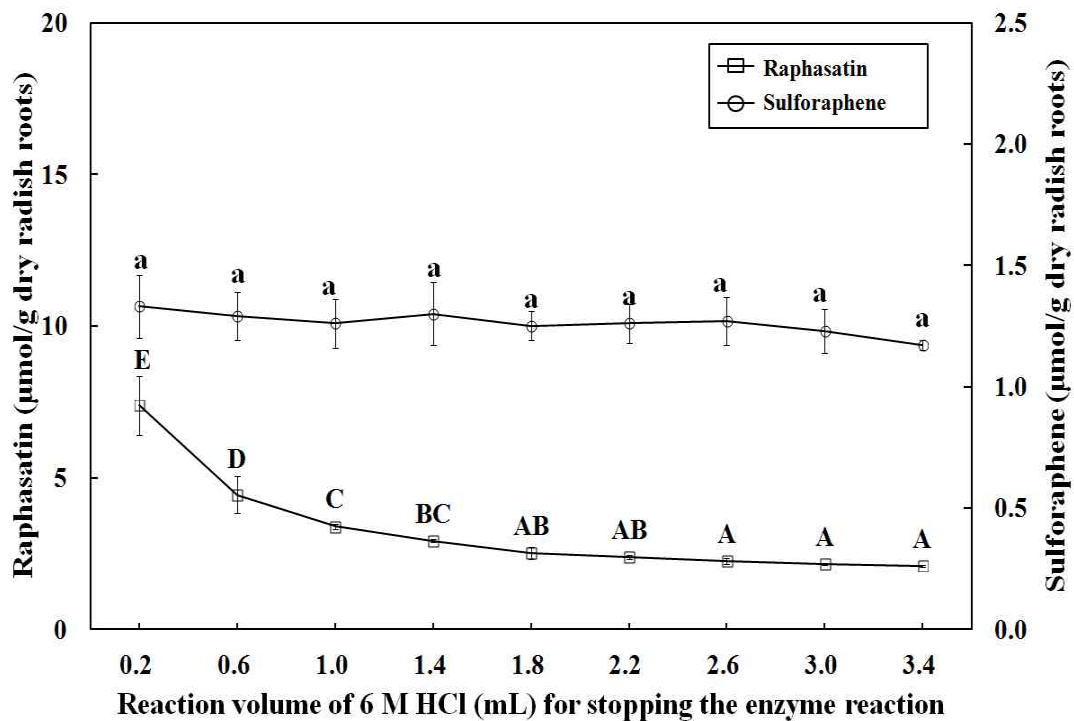
**Fig. 2.** Raphasatin and sulforaphene concentrations in radish roots with different reaction volumes of 6 M HCl at 15 min to stop the enzyme reaction. Each data is the mean of three replicates. Values followed by different letters are significantly different ($p$ <0.05).

**Fig. 3.** Raphasatin andwith different reaction times with 1.8 mL of 6 M HCl to stop the enzyme reaction. Each data is the mean of three replicates. Values followed by different letters are significantly different ($p < 0.05$).
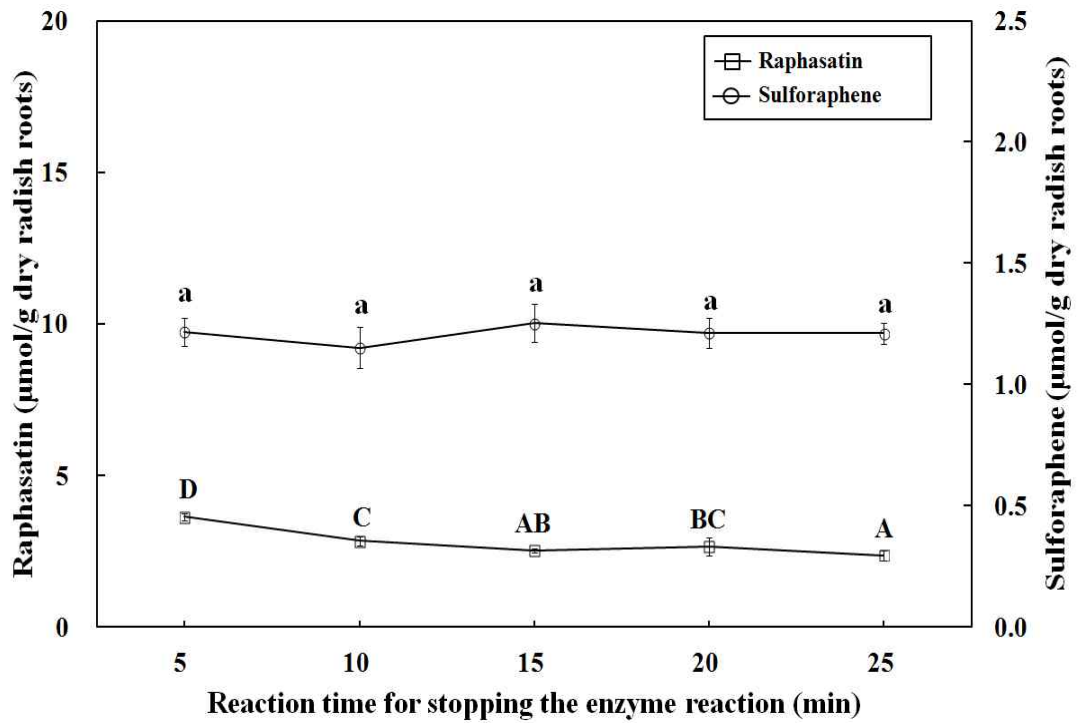
*3.3. Effect of polar and non-polar solvents on the formation of raphasatin and sulforaphene*

Radish powder and distilled water were mixed and incubated without shaking at 25°C for 5-25 min and then the amount of ITC was measured at various time points (Fig. 4). For 25 min, the yield of raphasatin was almost constant at about 2.52 µ mol/g of dry radish regardless of the hydrolysis time, while the amount of sulforaphene increased from 1.25 µmol/g of dry radish at 5 min to 1.73 µmol/g of dry radish at 10 min and then remained unchanged.

Production of sulforaphene from glucoraphenin, present in small amount in radishes, was completed within 10 min at 25°C. However, glucoraphasatin was not completely hydrolyzed into raphasatin from the large amount of glucoraphasatin present in radishes, and was not produced and degraded simultaneously during hydrolysis in water, because the yield of raphasatin was low (2.52 µmol/g of dry radish). Raphasatin is hydrophobic and unstable in aqueous or methanolic environments (Montaut et al., 2010). Therefore, for maximum sulforaphene production, hydrolysis in a polar medium (water) for 10 min without shaking should be used in further studies. However, for raphasatin, a polar and non-polar medium should be used in the hydrolysis process.

To increase the yield of raphasatin by minimizing its degradation during hydrolysis, a non-polar solvent (dichloromethane) (10 mL) was added to the radish powder (0.5 g) together with distilled water (8 mL), and the mixture was incubated without shaking at 25°C; the amount of ITC was measured after the indicated times (Fig. 5). The amount of raphasatin gradually increased from 9.17 µmol/g of dry radish after 5 min to 12.72 µmol/g of dry radish after 15 min and remained unchanged thereafter. The highest yield of raphasatin was obtained after 15 min, with almost complete transformation (94%) of glucoraphasatin in radishes (Table 1) due to the action of endogenous myrosinase. The amount of sulforaphene also gradually increased from 0.67 µmol/g of dry radish at 5 min to 1.39 µmol/g of dry radish at

15 min and then remained constant thereafter.

Because of the presence of dichloromethane in water during hydrolysis, the yield of raphasatin in radishes (12.72 μmol/g of dry radish) (Fig. 5) was increased by fivefold compared to that in water alone (2.52 μmol/g of dry radish) (Fig. 4). Therefore, the non-polar raphasatin produced by hydrolysis in water migrated to the non-polar dichloromethane medium and was protected. The yield of sulforaphene by the hydrolysis in a water and dichloromethane mixture (1.39 μmol/g of dry radish) decreased by about 20% compared to that in water alone (1.73 μmol/g of dry radish) due to the use of a mixture of polar and non-polar solvent as a reaction medium, which did not favor the polar sulforaphene. Therefore, to increase the formation and to minimize the degradation of non-polar raphasatin in an aqueous medium, hydrolysis should be performed in a mixture of polar and non-polar medium to get the maximum yield of raphasatin.
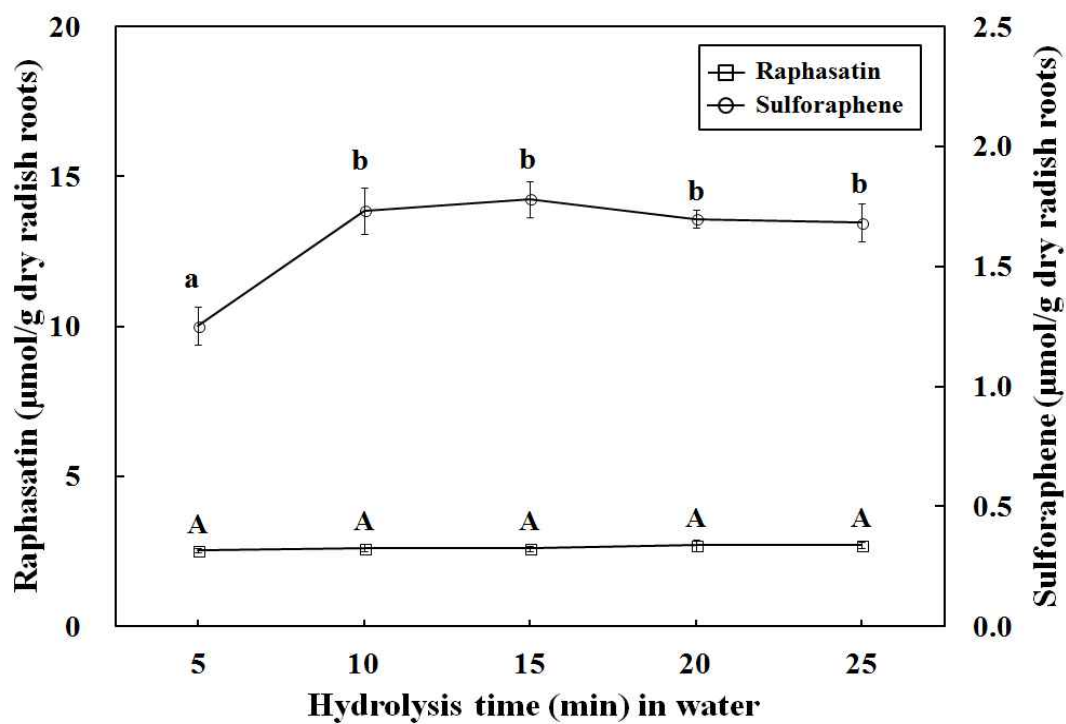
**Fig. 4.** Raphasatin and sulforaphene formation in radish roots during hydrolysis in water without shaking for 25 min. Each data is the mean of three replicates. Values followed by different letters are significantly different ($p < 0.05$).

**Fig. 5.** Raphasatin and sulforaphene formation in radish roots during hydrolysis in water and dichloromethane without shaking for 25 min. Each data is the mean of three replicates. Values followed by different letters are significantly different ($p$ <0.05).
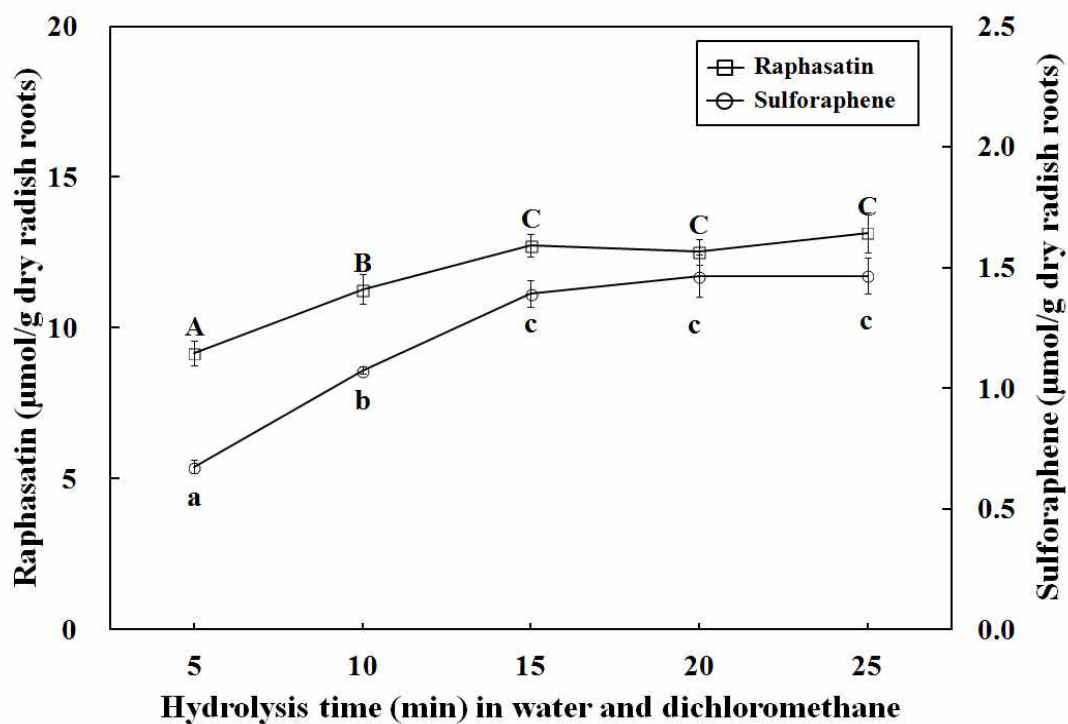
*3.4. Effect of shaking during hydrolysis on the formation of raphasatin and sulforaphene*

To provide more favorable conditions for migration of the raphasatin produced during hydrolysis into a non-polar medium, radish powder, distilled water, and dichloromethane were mixed and hydrolyzed with myrosinase, shaking in a water bath at 25°C for a controlled length of time (Fig. 6). The yield of raphasatin after 15 min of hydrolysis was increased by about 7% with shaking (13.57 µmol/g of dry radish) compared to without shaking (12.72 µmol/g of dry radish) (Fig. 5). This was because during hydrolysis with shaking, the non-polar raphasatin produced moved easily into a non-polar medium (dichloromethane) and retained its stability, and/or the shaking during hydrolysis provided greater opportunity for endogenous myrosinase to come into contact with glucoraphasatin in radishes. The sulforaphene yield was not changed regardless of use of shaking.

Based on the above experiments, the formation and minimization of the degradation of the non-polar raphasatin was favored in the hydrolysis reaction with the mixed polar and non-polar medium and shaking for 15 min. In contrast, for the polar sulforaphene, the optimal hydrolysis reaction conditions were the use of a polar medium for 10 min without shaking.

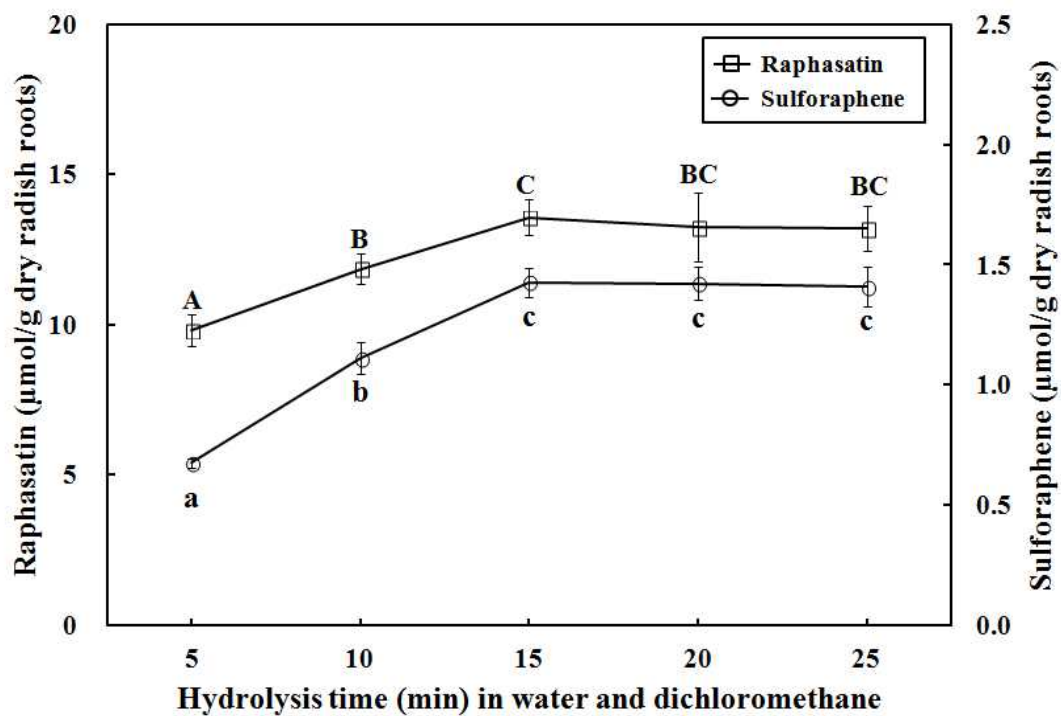**Fig. 6.** Raphasatin and sulforaphene formation in radish roots during hydrolysis in water and dichloromethane with shaking. Each data is the mean of three replicates. Values followed by different letters are significantly different ($p < 0.05$).

*3.5. Maximization of raphasatin and sulforaphene production by concurrent hydrolysis in water and in dichloromethane*

To simultaneously maximize the formation and maintain the stability of raphasatin and sulforaphene, radish powder and distilled water were mixed and hydrolyzed without shaking at 25°C for 10 min. Dichloromethane was then added and the mixture was further hydrolyzed with shaking in a water bath at 25°C for 15 min, before ITC yields were measured (Table 2).

The yield of raphasatin in the hydrolysis without shaking in water for 10 min and then with shaking in dichloromethane for 15 min (12.89 µmol/g of dry radish) was not significantly different from that in the hydrolysis only in water and then in dichloromethane with shaking for 15 min (13.57 µmol/g of dry radish, Fig. 5). However, the yield of sulforaphene in the hydrolysis without shaking in water for 10 min and then with shaking in dichloromethane for 15 min (1.93 µmol/g of dry radish) was about 12% greater than that of the hydrolysis only in water without shaking for 10 min (1.73 µmol/g of dry radish, Fig. 4). The yields of raphasatin and sulforaphene were not significantly different with and without shaking (12.46 µmol/g and 1.93 µmol/g of dry radish, respectively) in dichloromethane in the second hydrolysis process.

Based on the above experiments, it was concluded that to maximize the formation and minimize the degradation of raphasatin and sulforaphene, the endogenous enzyme hydrolysis of radishes should be performed in water for 10 min without shaking and then in dichloromethane for 15 min, with or without shaking.

**Table 2.** Raphasatin and sulforaphene formation in radish roots during concurrent hydrolysis in water without shaking and in dichloromethane with shaking.

| Hydrolysis method | Concentration (μmol/g of dry radish roots) | |
|---|---|---|
| | Raphasatin | Sulforaphene |
| Without shaking for 10 min in water and with shaking for 15 min in dichloromethane | $12.89 \pm 0.66^{A}$ | $1.93 \pm 0.07^{a}$ |
| Without shaking for 10 min in water and without shaking for 15 min in dichloromethane | $12.46 \pm 0.19^{A}$ | $1.93 \pm 0.00^{a}$ |

Values followed by the same letters are not significantly different ($p < 0.05$).

## 3.6. Effect of hydrolysis temperature on the formation of raphasatin and sulforaphene

The yields of raphasatin and sulforaphene at 25°C were slightly higher than at 37°C, but not significantly different from those at 10°C (Fig. 7). Generally, a temperature too low or too high is not beneficial for enzymatic activity. In our particular case, the optimum temperature for endogenous enzyme action appeared to be 25°C, where the maximum yields of 12.89 µmol/g of dry radish for raphasatin and 1.93 µmol/g of dry radish for sulforaphene were observed for the enzymolysis of GLs in radishes. Shen et al. (2010) investigated the conversion of glucoraphanin to sulforaphane using an endogenous myrosinase in broccoli seed powders over a temperature range between 5 and 55°C, with the maximum transformation achieved at 25°C.
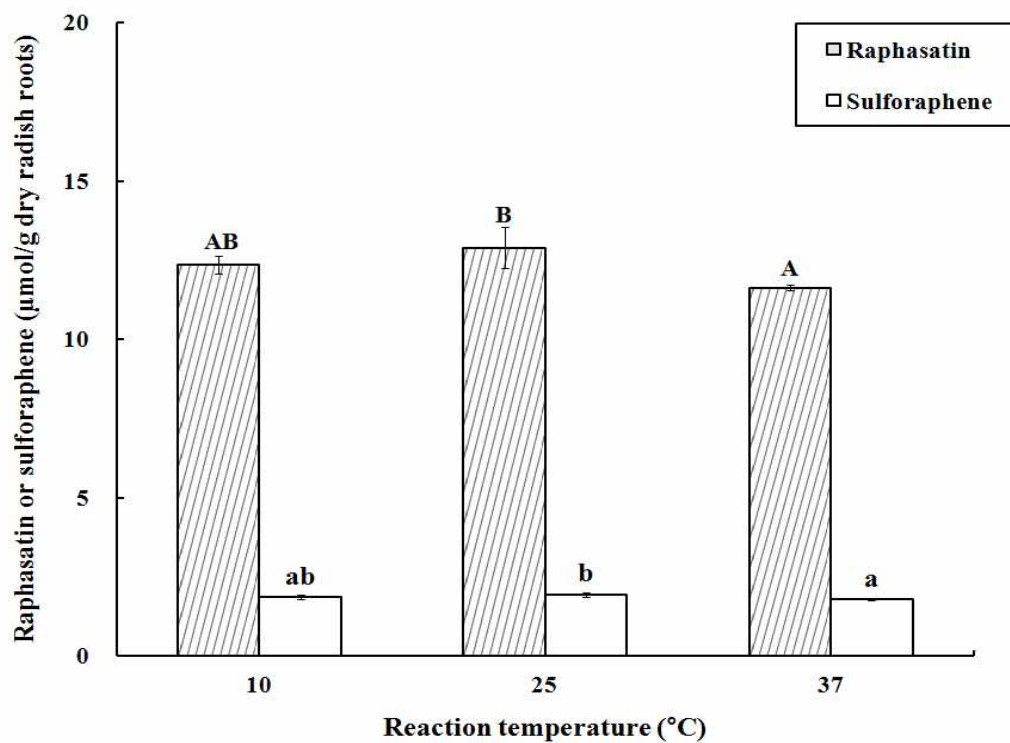
**Fig. 7.** Effects of hydrolysis temperature on raphasatin and sulforaphene formation in radish roots. Each data is the mean of three replicates. Values followed by different letters are significantly different ($p < 0.05$).

*3.7. Stability of raphasatin and sulforaphene during storage*

Because raphasatin is a significantly more potent activator of detoxification enzymes than its degradation products (Scholl et al., 2011), the stability of raphasatin and sulforaphene in radishes is crucial to their health effects during dietary consumption.

The hydrolyzed products of radishes were stored at 25°C for 10 days and ITC concentrations were measured at 2 day interval (Fig. 8). The raphasatin content decreased gradually as the storage period increased. After 2 days of storage at 25°C, the raphasatin content was decreased by only 17.6%, but after 10 days the content had decreased by 43.5%.

Scholl et al. (2011) found that the transformation of glucoraphasatin into raphasatin was initiated by the addition of water to freeze-dried radish powders, and after 24 h, raphasatin was degraded completely. In our experiment, more than 56% of the raphasatin remained after 10 days of storage at 25°C because the storage medium was the mixed hydrophilic and hydrophobic medium (water and dichloromethane) compared to the water used by Scholl et al. (2011). Therefore, to minimize the breakdown of raphasatin produced by hydrolysis, the hydrolyzed radishes should be stored in a mixed polar and non-polar medium (e.g., water and oil).

For sulforaphene, after 2 days of storage at 25°C, the content decreased by only 11.9% and then remained unchanged for 10 days. Scholl et al. (2011) found that upto 24 h after the initial hydrolysis, 77.6% of sulforaphene produced by the hydrolysis of radishes in water was remained. Matera et al. (2012) also found that under refrigerated conditions, only about 7% of the original ITCs content in radish sprout juices were lost in 12 months, although the stability tests were not performed at room temperature.

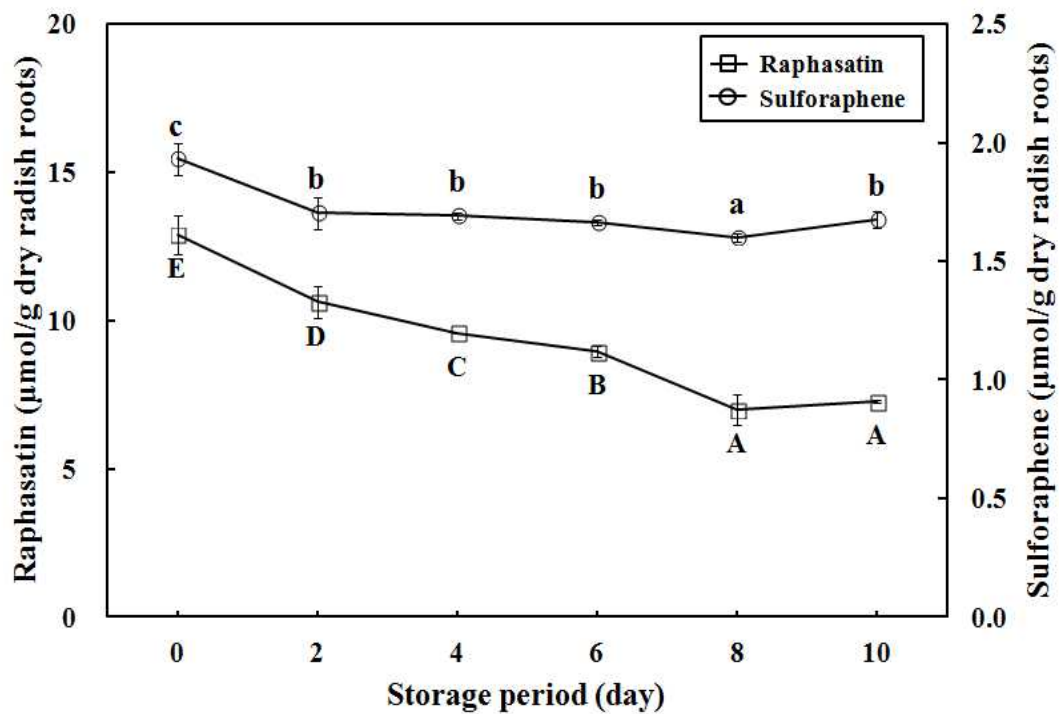**Fig. 8.** Stability of raphasatin and sulforaphene formed by endogenous enzyme hydrolysis during storage at 25°C for 10 days. Each data is the mean of three replicates. Values followed by different letters are significantly different ($p < 0.05$).

# 4. Conclusions

The biologically active compounds raphasatin and sulforaphene are formed during the hydrolysis of glucosinolates in radishes by an endogenous myrosinase. Raphasatin is very unstable, and is generated and simultaneously degraded to less active compounds during hydrolysis in aqueous media.

This study determined the hydrolysis conditions for maximizing the formation of raphasatin and sulforaphene by an endogenous myrosinase, and minimizing their degradation during the hydrolysis in radish roots. The endogenous enzyme reaction parameters, such as the reaction medium, reaction time, the type of mixing, and reaction temperature were optimized. A stability test for raphasatin and sulforaphene was also performed during storage of the hydrolyzed products at 25°C for 2, 4, 6, 8, and 10 days.

The formation and breakdown of raphasatin and sulforaphene in radish roots by endogenous enzymolysis was strongly influenced by the reaction medium, reaction time, and type of mixing. The production and stabilization of raphasatin in radishes was efficient in water and dichloromethane (a mixed hydrophilic and hydrophobic medium) with shaking for 15 min at 25°C. For sulforaphene in radishes the favorable conditions were water (the hydrophilic medium) as a reaction medium without shaking for 10 min at 25°C.

The maximum yields of raphasatin and sulforaphene were achieved in a concurrent hydrolysis reaction without shaking in water for 10 min and with shaking in dichloromethane for 15 min at 25°C. Under these conditions, the yields of raphasatin and sulforaphene were 12.89 and 1.93 µmol/g of dry radish, respectively. The stabilities of raphasatin and sulforaphene in the hydrolyzed products were 56.4 and 86.5% after 10 days of storage in water and dichloromethane at 25°C.

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# 국문요약


    무의 기능성 성분인 raphasatin과 sulforaphene은 무 중의 glucoraphasatin과 glucoraphenin이 내생효소(myrosinase)에 의해 가수분해되어 생성된다. Raphasatin은 지용성 성분으로 수용액상에서 매우 불안정하여 가수분해반응 동안 생성 및 파괴되지만 sulforaphene은 수용액상에서 비교적 안정하다. 따라서 본 연구에서는 무의 가수분해반응에 의해 raphasatin과 sulforaphene의 생성을 최대화하면서 파괴를 최소화하기 위한 최적의 가수분해 조건을 찾고자 하였다. 가수분해 반응조건으로 반응용매, 반응시간, 혼합방법, 반응온도를 최적화하였으며 가수분해반응 후 생성된 raphasatin과 sulforaphene을 25℃에서 10일간 저장하면서 안정성 실험을 수행하였다. Raphasatin과 sulforaphene의 생성과 파괴는 반응용매, 반응시간, 혼합방법에 의해 큰 영향을 받았다. Raphasatin은 물과 dichloromethane의 존재 하에 25℃에서 15분 동안 교반하였을 때 최대로 생성되었다. Sulforaphene은 반응용매로 물에서 25℃, 10분간 정치 하에 가수분해하였을 때 최대로 생성되었다. Raphasatin과 sulforaphene의 최대생성 가수분해반응 조건은 반응온도 25℃에서 물을 첨가하여 10분간 정치한 후 dicholoromethane을 가하여 15분간 교반하는 조건이었다. 이 조건에서 raphasatin과 sulforaphene의 최대 수율은 각각 12.89 μmol/g와 1.93 μmol/g이었다. Raphasatin과 sulforaphene의 25℃ 저장 중 안정성은 물과 dichlorometane의 존재 하에 10일 후 각각 56.4%와 86.5%가 잔존하였다.

# 감사의 글

포부를 가지고 대학원에 들어 온지 엊그제 같은데 이제 사회로 나갈 시기가 되니 아쉬움과 설레는 마음이 듭니다. 그동안 힘들 일도 있었지만 얻은 것이 많았던 대학원 생활을 마치기까지 많은 분들의 도움이 있었기에 감사의 마음을 전하고자 합니다.

부족한 저를 학문의 길로 이끌어 주시고 실험실 생활 시작부터 논문 마지막까지 세심한 지도와 항상 적극적인 행동의 마음가짐으로 많은 가르침을 주신 임상빈 지도교수님께 존경과 무한한 감사를 드립니다. 또한, 바쁘신 일정 중에도 저의 논문심사를 맡아 세심한 지도와 아낌없는 조언을 해주신 이동선 교수님, 김현정 교수님께도 마음 속 깊은 감사를 드리며, 학부부터 대학원까지 학문을 가르쳐 주신 고영환 교수님, 박은진 교수님, 홍근표 교수님께도 진심으로 감사함을 전합니다.

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