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연구논문

저장기간 중 돌산갓피클에서 Glucosinolates의 ACE 및 α-glucosidase 저해활성

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ACE and α-glucosidase Inhibitory Activity of the Glucosinolates in Dolsan Leaf Mustard Pickle during Storage

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Abstract: This study was carried out for investigate that physiological activity, quantification and qualitative were sinigrin of Dolsan leaf mustard pickle (DLMP) during storage. sinigrin contains high amounts of nutritional and medicinal compounds, which are important for maintaining optimum health. ACE inhibitory activity was ranged between 43.2 and 79.4%. DLMP methanol extracts demonstrated highest ACE inhibitory activity at 79.4% on day 14, whereas DLMP ethanol extracts demonstrated highest Angiotensin I-converting Enzyme (ACE) inhibitory activity of 43.2% at day 0. The α-glucosidase inhibitory activity of positive control 0.02% (v/v) acarbose was 78%. The DLMP methanol extracts had the highest α-glucosidase inhibitory activity at 64.0% on day 14, whereas DLMP ethanol extracts had the lowest α-glucosidase inhibitory activity of 42.8% at day 28. Sinigrin was high in DLMP methanol extracts at 49.55 μg/ml on day 14 of storage. Sinigrin standard was eluted at 2.73 min and MS analysis was m/z 283.03 along with fragment ions at m/z 204 and 149.06. These data show that sinigrin formed desulfo-glucosinolates $[M\text{-}SO_3\text{-}2H_2O+K+2H]^+$. Sinigrin concentration increased until day 14 and then decreased after that. DLMP methanol extracts had consistently higher sinigrin concentration than DLMP acetonitrile extracts during 28 days of storage.

Keywords: Dolsan leaf mustard, Pickle, Sinigrin, Glucosinolates, LC-PDA/MS/MS

1. INTRODUCTION

These days, people consume food products that are not only delicious bust also healthy, such as cruciferous vegetables, which are healthy foods known for their high content of glucosinolates. Glucosinolates are found in *Brassica* crops and are known to decrease cancer risk. Leaf mustard (*Brassica juncea*) is a member of the Brassicaceae family, and the mustard seeds are used as spices. The Dolsan leaf mustard is larger in size than the traditional leaf mustard, and harbors in high glucosinolates, polyphenols, and sulfur compounds (1).

Angiotensin I-converting enzyme (ACE) is a dipeptidyl carboxypeptidase that plays an important role in human renin-angiotensin-aldosteron system (RAAS). RAAS is a major regulator in human physiology. It controls blood pressure, volume and electrolytes and affects the heart, vasculature and kidney. ACE converts the inactive decapeptide angiotensin I into the potent

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vasoconstricting octapeptide angiotensin II the by cleaving dipeptide from the C-terminus into. This potent vasoconstrictor is also involved in the release of a sodium-retaining steroid, aldosterone, from the adrenal cortex, which has a tendency to increase blood pressure. ACE also catalyzes the degradation of bradykinin, a blood pressure lowering nonapeptide (2). Inhibition of ACE is considered as a useful therapeutic approach in the hypertension treatment, thus, in the development of drugs to control high blood pressure, ACE inhibition has become an important target. A large number of highly potent and specific ACE inhibitors have been developed as orally active drugs that are used in the treatment of hypertension and congestive heart failure (2). However, these synthetic ACE inhibitors are known to have irritating side effects such as dry cough, significant drop in energy, and angioedema. Therefore, ACE inhibitory peptides from food sources are commonly accepted as more advantageous over synthetic drugs (2,3).

Diabetes mellitus affects some 250 million people worldwide and is the leading cause of blindness, kidney failure, and amputation among adults (4). One of the major goals while treating patients with diabetes mellitus has been to achive blood glucose levels as close to normal as, as high blood glucose level is implicated in the development of diabetes associated macrovascular- and microvascular complications (5). However, in clinical practice, normalizing blood glucose levels is a formidable challenge, and even more difficult is the control of postprandial hyperglycemia (PPHG). Both dietary and pharmacological tools are now available for the management of PPHG. The pharmacological agents with the greatest effect on PPHG include insulin lispro, amylin analogues, and alpha-glucosidase inhibitors (6). The enzyme alpha-glucosidase catalyzes the final step in the digestive process of carbohydrates; hence, alpha glucosidase inhibitors could retard the digestion of dietary carbohydrates to suppress PPHG (7). Alpha-glucosidase inhibitors such as acarbose, miglitol, and voglibose are known to reduce PPHG primarily by interfering with the carbohydrate-digesting enzymes and delaying glucose absorption (6).

In recent years, there has been considerable interest in the potential for using natural food components as functional foods to treat hypertension, especially for people with borderline to mild hypertension that does not warrant the prescription of anti-hypertensive drugs (8). In this study, Dolsan leaf mustard pickle (DLMP) extracts were for α-glucosidase and ACE inhibitory activities. In addition, qualitative and quantitative was conducted for the DLMP extracted sinigrin of glucosinolates by using liquid chromatography with photodiode array and tandem mass spectrometry (LC-PDA/MS/MS).

2. MATERIALS AND METHODS

2.1. Materials

Dolsan leaf mustard was produced at Yeosu-si Dolsan-eup, Korea and harvested in October 2015. The harvested Dolsan leaf mustard was blanched at 80°C for 10 s. DLMP samples were prepared with Dolsan leaf mustard (65%), soy (10%), apple vinegar (7%), water (12%), garlic (1%) and condiment sauce (5%), and stored at 4°C for 28 days after preparation. DLMP samples were analyzed at 7 day intervals during the 28 days of storage.

2.2. Reagent

Rabbit lung acetone powder (ACE-8.2 units/g), Hippuryl-L-Histidiyl-L-Leucine (Hip-His-Leu), α-glucosidase, p-Nitrophenyl-α-D-glucopyranoside (p-NPG) and acarbose were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of high grade purity.

2.3. Preparation of DLMP extracts

DLMP samples were ground to particle sizes of 5-10 mm using a high speed mixer (Blender, Hanil, HNF-340, Seoul, Korea). For acetonitrile extraction, DLMP samples (10 g) were subjected to Soxhlet extraction with 200 mL of 50% (v/v) acetonitrile in a round-bottom flask at 93-94°C. For both methanol and ethanol extraction, DLMP samples (10g) were subjected to Soxhlet extraction with 200 mL of 80% (v/v) and methanol, 80% (v/v) ethanol in a round flask extracted at 60°C for 24 h. After cooling at room temperature 25°C, the extracts were filtered through Whatman No. 2 filter paper. The filtrate was then evaporated by an evaporator (EYELA, Tokyo, Japan) at 60°C and transferred to a lyophilized freeze-drying tube. The dried extracts were weighed and stored at 0°C prior to analysis.

2.4. ACE inhibition assay

ACE inhibitory activity was measured for each extract by the method of Cushman and Cheung (9) with modifications. The reaction mixture contained 100 μL of 5 mM Hip-His-Leu as a substrate, 100 μL of ACE solution (0.2 units/mL) in 0.1 M sodium borate buffer (pH 8.3), and 50 μL of DLMP sample solution. The reaction was carried out at 37°C for 30 min, and then terminated by adding 250 μL of 1 N HCl and 1.5 mL of ethyl acetate. After centrifugation, 1 mL of the supernatant that contained hippuric acid released Hip-His-Leu which was transferred into a test tube, and evaporated in dry oven (105°C) for 40 min. Then, the hippuric acid residue was dissolved in 1 mL of distilled water that the absorbance was measured at 228 nm with a spectrophotometer (SECOMAM, Ales, Fance). The ACE inhibitory activity was calculated by the following equation:

ACE inhibitory activity (%) = [(C – S) / (C – S')] × 100.

C is absorbance of control (distilled water);
S absorbance of sample;
S' the absorbance of control sample (the reaction was stopped by HCl after enzyme addition).

2.5. α-glucosidase inhibition assay

The assay was conducted for each extracts (300 μL) in 96-well plates (SPL Lifescience Co., Pocheon, Korea) with a microplate reader (UVM340, Biochrom, Cambridge, UK) according to procedures previously described (10). Briefly, the enzyme (α-glucosidase) and substrate (p-NPG) were dissolved in 67 mM sodium phosphate buffer (pH 6.8) at 0.5 U/mL and 5 mM, respectively. Four types of wells were made. Test wells contained 112 μL of buffer, 20 μL of enzyme, and 8 μL of sample. Test blank wells contained 132 μL of buffer and 8 μL of sample. Negative control wells contained 112 μL of buffer, 20 μL of enzyme, and 8 μL of DMSO. Negative blank wells contained 132 μL of buffer and 8 μL of DMSO. The positive control was used 0.02% (v/v) acarbose. The solutions were thoroughly mixed by careful shaking and maintained at 37°C for 15 min. After which was added to 20 μL of p-NPG each well, and the plates were incubated at 37°C for another 15 min. Finally, 80 μL of 0.2 M sodium carbonate in phosphate buffer was added to terminate the reaction. The amount of p-nitrophenol released from p-NPG by α-glucosidase hydrolysis was quantified by measuring its optical density (OD) at 405 nm.

Inhibition rate (%) = [1 – (OD test – OD test blank)] / (OD negative blank – OD blank) × 100

2.6. Quantitative analysis of sinigrin in DLMP with HPLC

Sinigrin standards were accurately weighed and dissolved in methanol. Calibration curves were made for each standard with 4 different concentrations (10, 25, 50 and 100 μg/mL). Mean areas (n=3) generated from the standard solutions were plotted against concentration to establish calibration equations. Sinigrin was extracted from DLMP and analyzed by HPLC using Phenomenex KINETEX C18 (150 mm × 2.1 i.d.; GL Science, Tokyo, Japan). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) with a gradient elution according to this program; 0-5 min 2% B; 5-30 min 35% B; 30-35 min 100% A. The total flow rate was 0.15 mL/min and monitored at an ultraviolet (UV) wavelength of 228 nm. The oven temperature was set at 40°C.

2.7. Qualitative analysis of Sinigrin in DLMP with LC-PDA/MS/MS

LC-PDA/MS/MS analysis was performed by Shimadzu Prominence UPLC with a Thermo Orbitrap XL system (Thermo Fisher Scientific, Bremen, Germany). The LC column used was Phenomenex KINETEX C18 (150 mm × 2.1 i.d.)(GL Science, Tokyo, Japan), and the mobile phase used was water (solvent A) and acetonitrile (solvent B) with a gradient of 0-32% for 30 min. The data acquisition time was 35 min, total flow rate was 0.94 mL/min and oven temperature was 40°C. Sinigrin concentration (ppm) was quantified by using a PDA detector to monitor the UV absorbance at 200-360 nm. Sinigrin (5% solution) was co-injected as an internal standard into each sample solution. MS and MS/MS data were obtained by electrospray ionization (ESI) in the positive ion mode with the parameters : m/z range 50-1,500, source voltage 5.40 kV, capillary voltage 48.00 V, capillary temperature 275°C, sheath gas flow 50 L/min, aux gas flow 10 L/min, source current 1,000 mA, and tube lens 100 V.

2.8. Statistical analysis

All tests and analyses were repeated at least three times. The results were expressed as mean±standard deviation (SD). One way analysis of variance (ANOVA) and Duncan's test were used for multiple comparisons with SPSS version 21.0 (SPSS Institute, Chicago, IL, USA). The values were considered as different significance that the *P* value was less than 0.05.

3. RESULTS AND DISCUSSION

3.1. ACE inhibitory activity

ACE inhibitory activity in DLMP extracts according to storage

Table 1. Angiotensin I-converting Enzyme (ACE) inhibitory activity of Dolsan leaf mustard pickle (DLMP) extracts

Extract	ACE inhibitory activity (%)				
	0[1)]	7	14	21	28
Methanol	67.41 ± 0.01^{Aa}[2)]	72.25 ± 0.02^{Ca}	79.43 ± 0.02^{Da}	75.32 ± 0.01^{Ea}	69.08 ± 0.03^{Ba}
Ethanol	43.24 ± 0.01^{Ab}	55.03 ± 0.03^{Cb}	63.75 ± 0.02^{Db}	53.01 ± 0.02^{Eb}	47.53 ± 0.02^{Bb}
Acetonitrile	58.41 ± 0.04A^{c}	63.78 ± 0.01^{Cc}	70.32 ± 0.01^{Dc}	65.57 ± 0.02^{Ec}	60.32 ± 0.03^{Bc}

[1)]Storage.

[2)]All values are mean±SD of the triplicate determination. Means in row (a-c) and a column (A-E) followed by different superscripts are significantly different at $p<0.05$ by Duncan's multiple range test.

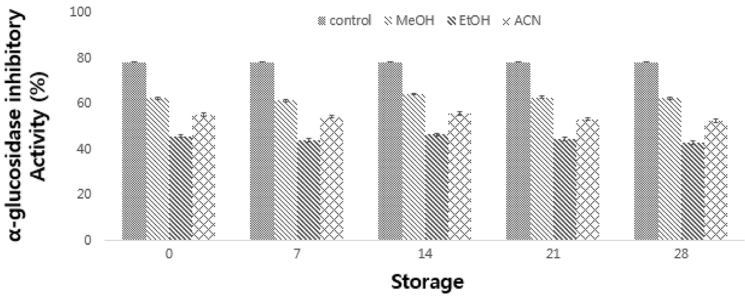

Fig. 1. α-Glucosidase inhibitory activity of Dolsan leaf mustard pickle (DLMP) extracts.

Table 2. Total sinigirn contents in Dolsan leaf mustard pickle (DLMP) extracts

Extract	Sinigrin contents (μl/mL)				
	0[1)]	7	14	21	28
Methanol	44.74 ± 0.01$^{Eb2)}$	45.12 ± 0.01^{Db}	49.56 ± 0.01^{Ab}	45.96 ± 0.01^{Cb}	43.96 ± 0.02^{Bb}
Acetonitrile	28.45 ± 0.02^{Ea}	28.94 ± 0.01^{Da}	35.88 ± 0.02^{Aa}	32.98 ± 0.01^{Ca}	30.74 ± 0.01^{Ba}

[1)]Storage.

[2)]All values are mean±SD of the triplicate determination. Means in row (a-b) and a column (A-E) followed by different superscripts are significantly different at $p<0.05$ by Duncan's multiple range test.

was showed in Table 1. ACE inhibitory activity was significantly different ($p<0.05$) among the DLMP extracts, values ranged from 43.2 to 79.4%. DLMP methanol extracts had the highest ACE inhibitory activity at 79.4% on day 14 and DLMP ethanol extracts had the highest ACE inhibitory activity at 43.2% on day 0. The results were consistent with those from a previous report (11) which saw that ACE inhibitory activity of Dolsan leaf mustard stem and leaf was 47.4% and 88.2%, respectively. Fermented Dolsan leaf mustard kimchi as well a variety of other foods have been reported for their ACE inhibition activity (12). Dolsan leaf mustard kimchi was the highest ACE inhibitory activity showed 76% during fermentation at day 12. DLMP methanol extracts had the higher ACE inhibitory activity than Dolsan leaf mustard stem of Dolsan leaf mustard kimchi.

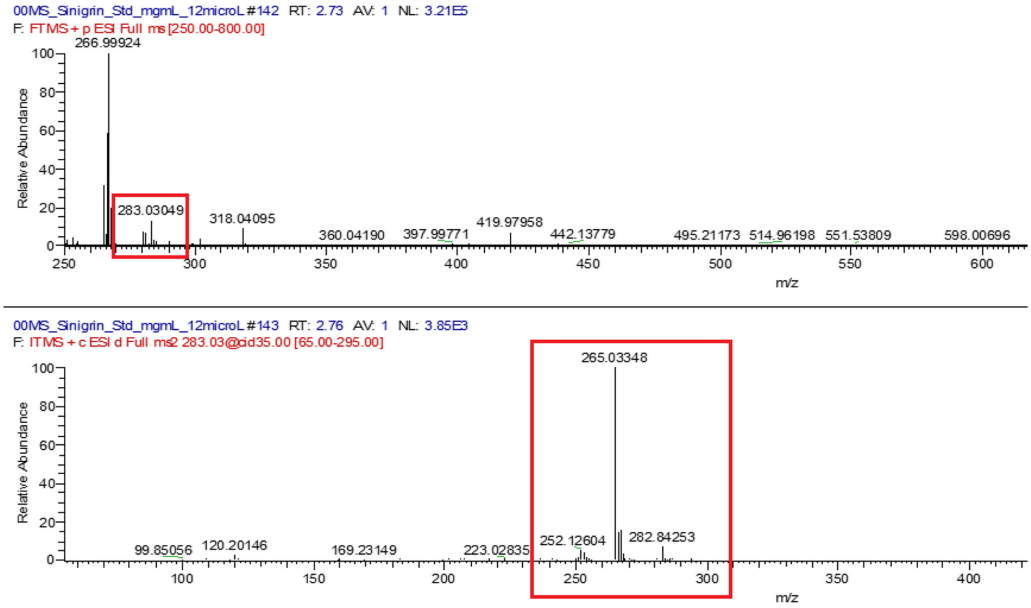

Fig. 2. MS and MS/MS analysis of sinigrin standard. Selected ion chromatography screened by m/z 279.2 and MS spectrum (top) of the 2.7 min component, and MS/MS of the m/z 283.03 component (bottom).

3.2. α-glucosidase inhibitory activity

α-glucosidase inhibitors that reduce postprandial hyperglycemia are key factors in the treatment of type 2 pre-diabetic states which can reduce the progression of diabetes (13). DLMP extracts that showed inhibitory activity against the enzyme ranged from 42.8 to 64.0% at a concentration of 5 mg/mL (Fig. 1). Furthermore, α-glucosidase inhibitory activity was significantly different (p<0.05) among the DLMP extracts. The α-glucosidase inhibitory activity of 0.02% (v/v) acarbose positive control was 78%. DLMP methanol extracts had the highest α-glucosidase inhibitory activity at 64.0% on day 14, whereas DLMP ethanol extracts had the lowest α-glucosidase inhibitory activity at 42.8% on day 28. Although lower than the synthetic antidiabetic agent acarbose, α-glucosidase inhibitory activity of Dolsan leaf mustard from previous study was measured at 32.4±2.6 (14). Based on the results, the high potency of DLMP methanol extracts was encouraged to justify identification of the compounds that was responsible for the α-glucosidase inhibitory effect.

3.3. Quantitative and qualitative analysis of sinigrin in DLMP extracts

After analyzing ACE and α-glucosidase inhibitory activities were higher in both methanol and acetonitrile extracts. Therefore, methanol and acetonitrile extracts were analyzed quantitatively and qualitatively for sinigrin. Based on HPLC screening, sinigrin was highest among the diverse group of glucosinolates. There was a high sinigrin concentration in DLMP methanol extracts at 49.55 μg/mL during storage on day 14 (Table

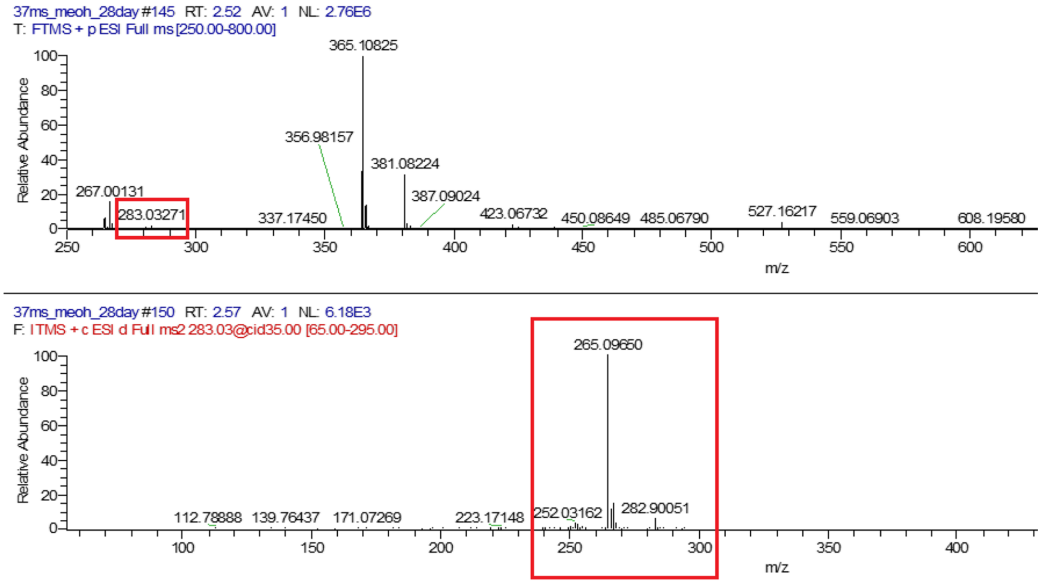

Fig. 3. MS and MS/MS analysis of the Dolsan leaf mustard pickle (DLMP) methanol extract. These fragment patterns completely corresponded to those of sinigrin standard (desulfo-sinigrin).

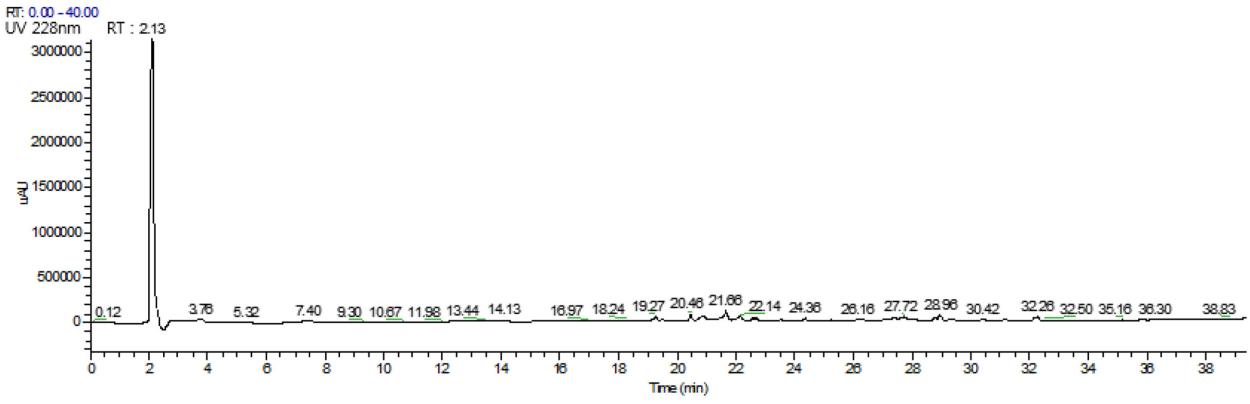

Fig. 4. LC chromatograms (UV 228 nm) of Dolsan leaf mustard (DLMP) methanol extract on day 14.

2). Sinigrin standard eluted at 2.73 min, and MS analysis peaked at m/z 283.03 along with the fragment ions at m/z 204 and 149.06 (Fig. 2). Therefore, these data showed that sinigrin formed desulfo-glucosinolates $[M\text{-}SO_3\text{-}2H_2O+K+2H]^+$. DLMP methanol extracted sinigrin was detected and confirmed by LC-MS/MS analysis (Fig. 3, 4). Although different solvent extracted methods was affect sinigrin concentration on day 14 (Table 2). Overall sinigrin concentration increased until day 14 and after decreased. DLMP methanol extracts showed consistently higher sinigrin concentration than DLMP acetonitrile extracts (during the 28 days of storage).

4. CONCLUSION

Sinigrin is generally distributed in aleurone cells and myrocin cell of *brassica juncea* vegetables; secreted with myrosinase during tissue destruction by pathogens. In other words, myrosinase could be released when storage of DLMP damaged plant, thus, it would have gradually decreased continued activation and breakdown. Myrosinase activies in radishes are low according to pH reports (15). The pH was lowered in DLMP after 15 days at 4°C (16) where the pH decrease weakened the activity of myrosinase; however, it was no a large differences between early and late (17). The glucosinolate breakdown products were isothiocyanate and sinigrin that was reported to the physiological activity (18). It is known that the effect of inhibiting hepatic tumor (19) and lung cancer (20).Therefore, the physiological and functional changes during storage are expected and influenced by sinigrin during secretion.

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