Glucosinalate compound and antioxidant activity of fresh green cabbage and fermented green cabbage products

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Abstract

This research focused on the investigation of the total phenolic contents (TPC), total flavonoid contents (TFC), antioxidant activity and analysis of glucosinolate compound in fresh green cabbage and fermented green cabbage products. The TPC and TFC of all samples were determined using folin-ciocalteu and aluminum chloride colorimetric methods, respectively. The antioxidant activity was assessed using 2,2- diphenyl- 1- picrylhydrazyl (DPPH) free radical scavenging method. The results showed that all fermented green cabbage products exhibited the total phenolic and flavonoid contents and antioxidant activity significantly lower than those from fresh green cabbage. The analysis of glucosinolates active compounds namely sinigrin, progoitrin, glucotropaeolin and glucoraphanin in samples was investigated via high performance liquid chromatography (HPLC) technique. The result showed that only sinigrin was detected in both fresh green cabbage and fermented green cabbage products, particularly in fresh green cabbage with a highest amount.

Keywords: glucosinolate, antioxidant activity, sinigrin, phenolic compounds

Introduction

Free radicals have been implicated in the development of a number of various diseases such as cancer, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases and inflammation, giving rise to the studies in antioxidants for the prevention and treatment of diseases. Generally, human bodies have their own substances that can help to get rid of free radicals. Most living organisms possess enzymatic and non-enzymatic antioxidant defence and repair systems that have evolved to protect them against oxidative stress. However, these native antioxidative systems are generally not enough to prevent the living organisms from oxidative damage [Li S. *et.al.*, 2012; Lobo V. *et.al.*, 2010]. Therefore, there is a great demand for natural antioxidants in many plants and food materials, which enhances health and food preservation. Many researches demonstrated that various vegetables have a lot of natural antioxidants which can prevent and kill many types of

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cancer cells and one of those vegetables is the brassica vegetables. Vegetable belonging to the family Brassicaceae are widely consumed by humans. Several authors report that Brassica vegetables are high in natural antioxidants and consumption of these vegetables is associated with reduced risk of cancer in humans [Malan R. *et.al.*,2011; Unal K. *et.al*., 2014]. In addition, recent studies show that ingestion of Brassica vegetables may decrease the incidence of different types of cancer. Brassica vegetables showed the existence of many antioxidant compounds such as glucosinolates, phenolic acids and flavonoids. Phenolic acids and flavonoids are efficient antioxidants and exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic and vasodilatory effects. In particular, brassica vegetables have been reported as rich sources of glucosinolate, which is a class of plant secondary metabolite, usually formed as the potassium or sodium salt [Breinholt V., 1999; Rameeh V., 2015; Radoševic K. *et.al.*, 2017; Anandan S. *et.al.*, 2018). The common structural feature of intact glucosinolates is shown in **Figure 1**.

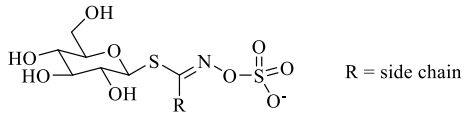

Figure 1 The structure of glucosinolates compound

Glucosinolates have long been known for their fungicidal, bacteriocidal, nematocidal, allelopathic properties and are fast gaining popularity because several studies have reported their ability to fight certain types diseases of cancers and other chronic and degenerative [Song L. *et.al.*, 2005; Totušek J. *et al.*, 2011; Francisco J. *et.al.*, 2016]. Green cabbage is the most common and best-known variety of brassica vegetables. They are widely consumed by humans as fresh and under different preservation technologies. The fermentation of green cabbage is one of the popular preservation technologies, which yields palatable food, which either retains a part of the original nutrients or new ones are formed, in a probiotic environment (lactic microorganisms). The fermentation can be preserved for long periods of time, without refrigeration, especially when herbs, spices and other ingredients are added to improve the stability, aroma and flavor [Fu S. *et.al.*, 2002; Vicas S. *et.al.*, 2015; Martinez-Villaluenga C. *et.al.*, 2009]. In addition, fermented Green cabbage is very popular in many ethnic cultures and being the major product of this category. Nowadays, the consumption of fermented green cabbage products is increasing. According to previous researches, the antioxidant activities and glucosinolate profiles from green cabbage have been studied during different cooking processes, such as blanching and treatment with acetic acid and thermal degradation [Wennberg M. *et. al.*, 2006; Song L. *et.al.*, 2007; Bongoni R. *et.al.*, 2014; Oerlemans K. *et.al.*, 2006; Jones R.B. *et.al.*, 2010]. Although the antioxidant activities and glucosinolate content as active compound of green cabbages have been studied widely because of their beneficial effects on health, the effect of fermentation processing on their bioactive compounds

is still not well investigated. In addition,there are no data about the level of antioxidant property and glucosinolate content from green cabbage after the fermentation. Therefore, the main aims of this paper were : (i) to investigate the impact of fermentation on the level of individual glucosinolate compounds, from fresh green cabbage and four different fermented green cabbage products including salty fermented, spicy and sweet fermented, sweet and sour fermented, salty and spicy fermented and rice-milk fermented products by using a high performance liquid chromatography (HPLC) technique and (ii) to determine anti-nutritional factors including antioxidant activity using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method and total phenolic acid and flavonoid contents *via* the folin-ciocalteu and aluminum chloride colorimetric methods, respectively. This data from this study might be greatly beneficial as informative base on developing accessions with enhanced health benefits for the people who consume fresh and fermented green cabbage.

Experimental

Plant materials and apparatus

Plant materials: Commercial fresh green cabbage and fermented green cabbage products including salty fermented, spicy and sweet fermented, sweet and sour fermented, salty and spicy fermented and rice-milk fermented products were purchased from supermarkets in Phitsanulok province, Thailand, in April 2019. The samples were stored at 4 ◦C until further analysis.

Chemicals and reagents: Aryl sulfatase enzyme and the standards of glucosinolate compounds including sinigrin, progoitrin, glucotropaeolin and glucoraphanin were from purchased from Sigma-Aldrich Corporation. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and gallic acid and quercetin were obtained from Fluka. The Folin-Ciocalteu's reagent and aluminum chloride hexahydrate were obtained from Merck. All solvents used were purchased from Labscan. All other chemicals and reagents were of analytical grade.

Apparatus: Spectrophotometric determinations were performed on a PerkinElmer 554 UV–VIS spectrophotometer with 1-cm path length cuvettes. HPLC analyses were performed on Agilent LC1200 Series HPLC with a diode array detector operating at wavelengths between 200 and 600 nm. The column was Inertsil ODS-3 (C18), 3.0 x 150 mm.

Preparation of fresh and fermented green cabbage samples

The edible parts of the samples were cut into small pieces and stored at -20 ºC prior to extraction.

Solvent extraction of samples to study in antioxidant activity and total phenolic and flavonoid contents

First, 10 g of samples were extracted with 10 ml of 1% of acetic acid in methanol for an hour at room temperature. The extracts were shaken for 30 min, centrifuged (3,000 rpm) for 15 min at room temperature. The extracts were filtered and the organic layer was collected. The extraction process was repeated twice and

the extracts from all the three washes were pooled. The extract was evaporated under pressure till all the solvent had been removed and further removal of water was carried out by freeze drying to obtain a dried crude extract. The crude extracts were stored at 4^{o}C for further analysis.

Determination of total phenolic contents (TPC)

Total phenolic content was studied using the folin-ciocalteu colorimetric method [Tarola A.M. et.al., 2013] Briefly, 0.5 mL of each crude extract (1 mg/mL) was made up to 1 mL with methanol, then mixed with 1.5 mL of the Folin–Ciocalteu reagent (diluted1:10, v/v) followed by 2 ml of Na_2CO_3 (7.5%, v/v) solution. The mixture was vortexed for 10 sec and allowed to stand for 40 min at room temperature for color development. Absorbance of the solution was measured at 765 nm wavelength and the results were expressed as mg/g gallic acid equivalent (GAE) based on the calibration curve of gallic acid standard solutions (10-70 ppm).

Determination of total flavonoid contents (TFC)

The aluminum chloride colorimetric method was used for the determination of the total flavonoid content of the sample as described [Rebaya A. *et.al.*, 2014]. For total flavonoid determination, quercetin (20 to 120 mg/mL) was used to make the standard calibration curve. An amount of 125 µL of each crude extract was added to 75 µL of a 5% $NaNO_2$ solution. After 5 min, 150 µL of aluminium chloride (10%) was added to the mixture, which was then allowed to stand for another 5 min. Then, 750 µL of NaOH (1 M) was added to the mixture, followed by the addition of 275 µL of deionized water. After mixing, the solution was incubated for 15 min at room temperature. After incubation, the mixture turned to pink and the absorbance was measured at 510 nm wavelength with a UV-Vis spectrophotometer.The concentration of total flavonoid content in the test samples was calculated from the calibration plot and expressed as gram of quercetin equivalents (QE) per 100 g of dried extract. all the determinations were carried out in triplicate.

Antioxidant activity testing

The antioxidant activity of samples was determined with the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay [Nooman A.K. *et.al*., 2008]. The final reaction mixture for the assay (3 mL) was prepared as follows: 1.5 mL of various concentrations of extract solutions were mixed with 1.5 mL DPPH solution (3×10^{-4} M) and the reaction mixtures were incubated in dark at room temperature for 1 h. Absorbance of the solution was measured at 517 nm wavelength having ethanol as a blank while BHT was used as positive control. Control was prepared by mixing 1 mL of ethanol with 2 mL of DPPH solution. Antioxidant capacity based on the DPPH free radical scavenging ability of extracts was calculated using the following equation:

$$\text{Percentage inhibition} = (1\text{-}A)/A_0) \times 100$$

where A_0 is the absorbance of the control reaction (containing all reagents except the extract), and A is the absorbance of the extract.

The antioxidant activity of each sample was expressed in term of the IC_{50} (μM required to inhibit DPPH radical formation by 50%), which was calculated from the log-dose inhibition curve.

Sample extraction for glucosinolate compounds determination

Glucosinolate compounds were extracted and analyzed as described by Lee [Lee M.K. *et.al.*, 2014]. Briefly, 100 g of samples were extracted with 70% methanol (1.5 mL) and heated on a heating block at 70ºC for 5 min. The extracts were cooled down and centrifuged for 10 min. After centrifugation, the supernatant was transferred to another tube and stored as the first portion. The extraction process repeated twice as described above. Once completed, the extracts from all three washes were collected and concentrated under vacuum to obtain dried glucosinolate extracts, which were stored at 4ºC for further analysis.

Desulfation of glucosinolate extracts

First, the ion exchange column (DEAE Sephadex A-25) was prepared. Briefly, the suspension of DEAE anion exchange resin (Sephadex A-25) in 0.5 M sodium acetate was transferred to a pipette column. The column was then rinsed with 1 ml DI water twice. One milliliter of the glucosinolate extract was transferred to a prepared column and aryl sulfatase enzyme solution (Helix pomatia, type H1) was added to the column (75 μl) and left to react for 16 h at ambient temperature. Then desulfoglucosinolate was eluted with three 1 ml portions of water and the effluent was filtered and analyzed for glucosinolate by HPLC.

The analysis of glucosinolate compounds using HPLC technique

The glucosinolate compounds were analyzed via Agilent LC1200 Series HPLC using a diode array detector. The sample (20 μl) was injected into the column and the signals were monitored at 227 nm. The desulfoglucosinolates were separated using a type C18 column (Inertsil ODS-3 C18 Type Size 3.0 mm × 150 mm, 3 μm) operated at 40ºC and elution was carried out using a gradient system consisting of water (A) and acetonitrile (B) at a flow rate of 1.0 mL/min. The total running time was 30 min with a gradient as follows: 100% A and 0% B for 5 min, then in 23 min to 0% A and 100% B and in 7 min back to 100% A and 0% B. Individual glucosinolates were identified in comparison with the retention time of standard compounds and areas of standard compounds including sinigrin, progoitrin, glucotropaeolin and glucoraphanin. The calibration curves of glucosinolate standards were established using known concentrations of the standard compounds.

Statistical analysis

The statistical analyses were carried out by ANOVA using the general linear model of SPSS 14.0 for Windows. All analyses was carried out in triplicate and expressed as mean ± SD to show variations in the various experimental data. P values less than 0.05 were considered statistically significant

Results and Discussion

Determination of total phenolic and flavonoid contents in fresh and fermented green cabbage products

The folin-ciocalteu colorimetric method was selected to investigate the total phenolic contents because this method is simple, sensitive, and precise [Serea C. *et.al*., 2011]. The total phenolic content was carried out based on the reaction of various sample solutions with the folin-ciocalteu reagent to yield blue colored solutions which maximum absorption at 750 nm. The absorbance was compared with the standard solutions of gallic equivalents (the standard curve equation: $y = 0.0043x – 0.004$, $r^2 = 0.9991$) and total phenolic contents were expressed as gallic acid equivalents (GAE) in mg/g crude extract. In case of, the total flavonoid content among the crude extracts was determined using spectrophotometric method with aluminum chloride colorimetric method. Aluminium chloride will form stable complex with carbonyl group at C4 and hydroxyls at C3 (flavonols) and C5 in flavonols and flavones. It could also form labile acid complexes with hydroxyls in the ortho position in B rings of flavonoids [Sembiring E. N. *et.al*., 2018].The content of flavonoids was expressed in term of quercetin equivalent (mg of QE/g of extract) using the standard curves equations ($y = 0.0049x + 0.0633$, $R^2 = 0.9990$). The total phenolic and flavonoid contents in samples were shown in **Table 1**.

Table 1. Total phenolic and flavonoid contents in fresh green cabbage and fermented green cabbage products.

Green cabbage Samples	Total phenolic contents (mg GAE/ g crude extract)	Total flavonoid contents (mg QE/ g crude extract)
Fresh	219.53±0.09	70.17±0.15
Salty fermented	45.81 ±0.11	24.70±0.22
Spicy and sweet fermented	31.16±0.17	22.06±0.19
Sweet and sour fermented	29.53±0.13	35.95±0.12
Salty and spicy fermented	31.86±0.17	16.54±0.19
Rice-milk fermented	178.37±0.21	46.57±0.15

From **Table 1**, the fermentation types change the content of total phenolic and flavonoid contents in the end products. Hence, the fermented green cabbage products provide a low content of total phenolic and flavonoid contents. The initial level of total phenolic and flavonoid contents in fresh green cabbage was 219.53 mg GAE/ g crude extract at a concentration of 1000 $\mu g.ml^{-1}$ and 70.17 mg QE/ g crude extract at a concentration of 1000 $\mu g.ml^{-1}$, respectively. It was found that all fermented green cabbage showed the total phenolic and flavonoid contents significantly lower than those from fresh green cabbage. The high loss of total phenolic and flavonoid contents was observed. This could be due to the decomposition of unstable phenolic compounds or losses during the fermentation process. In addition, the fermented

process might be led to enzymatic such as esterase and glycosidase degradation of the phenolic derivatives due to microorganisms involved in the fermentation process [Vicas S. I. *et.al.*, 2015]. According to previous research, factors such as pH, temperature, lactic acid formation and fermentation conditions can influence the remaining phenolic compounds in the samples [Martinez-Villaluenda C. *et.al.*, 2009; Ahmed F.A. *et.al.*, 2013]. The effect of fermentation conditions on total phenolic and flavonoid contents in white cabbage has been reported [Uzuki,C. S. *et.al.*, 2006]. It was found that total phenolic and flavonoid contents were highest in fresh cabbage while fermented white cabbage significantly decreased the contents of total phenolic and flavonoid compounds. These could support the result that there are highest of phenolic and flavonoid contents in fresh green cabbage while significantly decreased in fermented samples. Moreover, when comparing total phenolic and flavonoid contents in all fermented green cabbage products, it was found that the rice-milk fermented product exhibited the highest amounts of phenolic and flavonoid contents. It can be explained that rice-milk are the essential sources of phenolic and flavonoid compounds such as ferulic acid, coumaric acids, chlorogenic acid and gallic acid etc [Walter M. *et.al.*, 2011]. Therefore, these compounds might be diffusion into green cabbage, while fermented treatment which resulting rice-milk fermented product had higher total phenolic and flavonoid contents than those of another fermented green cabbage products.

Antioxidant activity of fresh green cabbage and pickled green cabbage products

Antioxidant activity of samples was determined by the DPPH radical scavenging method. This method provides an easy and rapid way to evaluate antioxidants. In DPPH scavenging assay, the antioxidant activity was measured by the decrease in absorbance of DPPH radical. The changing of color of DPPH radical can be quantitatively measured from absorbance (Abs) at 517 nm wavelength. The parameter IC_{50} is used for the interpretation of the results from the DPPH method and is defined as the concentration of substrate that causes 50% loss of the DPPH activity (color). Lower IC_{50} value indicates higher antioxidant activity in the extract. The antioxidant activity of all samples was expressed in terms of IC_{50} values (ppm) as shown in **Figure 2**.

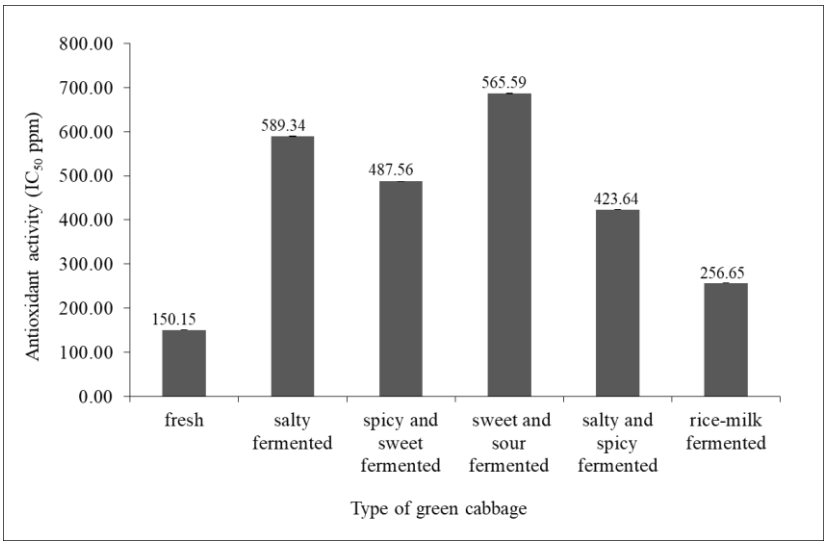

Figure2. The antioxidant activity of all samples was expressed in terms of IC_{50} values (ppm). The results are the average of triplicates ± SD.

From **Figure 2**, it was found that the highest radical scavenging activity was showed by fresh green cabbage (IC_{50}= 150.15 ppm) which is significantly higher than that of all fermented green cabbage products. The radical scavenging activity in samples decreased in the following order: rice-milk fermented > salty and spicy fermented > spicy and sweet fermented > salty fermented > sweet and sour fermented products. It was found that some active compounds were decomposed during the fermentation process. It can be explain that fermentation induces the structural breakdown of plant cell walls, leading to the decomposition of various antioxidant compounds. In addition, the effect of fermentation conditions such as pH, temperature and fermented duration can influence the antioxidant property in the samples. The comparison of antioxidant activity of all fermented green cabbage products was also investigated. It was found that rice-milk fermented product showed the best result with anti-radical activity in scavenging DPPH radical (IC_{50} = 256.65 ppm). This was attributed to the presence of antioxidant compounds such as phenolic acids, flavonoids, γ - oryzanol, tocopherols and tocotrienols in rice-milk [Rashid N. Y. *et.al.*, 2015] resulting fermented green cabbage in rice-milk showed good antioxidant properties. These results were corresponded to the study of total phenolic and flavonoid contents in all samples.

Quantification of glucosinolate compounds from fresh green cabbage and fermented green cabbage products

The analysis of glucosinolate compounds was carried out *via* HPLC technique for obtaining information on the glucosinolate compounds profiles. Four standard glucosinolate compounds used in this work included sinigrin, progoitrin, glucotropaeolin and glucoraphanin as their chemical structures shown in **Figure 3**. The choice of these compounds as standards is based on the availability of the stable

and pure substances. In addition, all of standard compounds were the most abundant and the major glucosinolate in cruciferous vegetables.

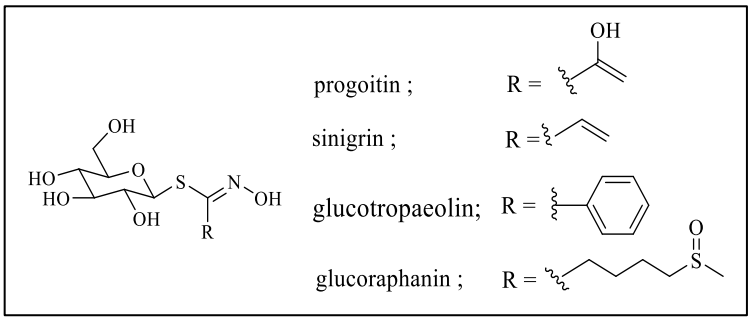

Figure 3 The structure of four standard glucosinolate compounds

The chromatographic conditions for the quantification of glucosinolate compounds were optimized by conducting preliminary trials with the standard mix of the glucosinolate compounds. It was found that the optimized HPLC conditions can clearly separate all standard desulfoglucosinolate compounds and the optimized time of the chromatographic run was 30 min as shown in **Figure 4**.

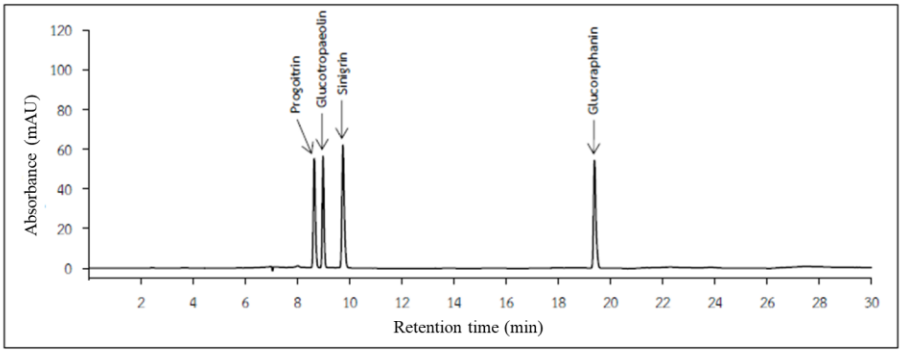

Figure 4. The HPLC chromatogram of four standard disulfoglucosinolate compounds detected at 227 nm

In addition, the calibration curves of all standard disulfoglucosinolate compounds were performed. Seven different concentrations of all standard active compounds in the range of 10–64 μg/mL were employed. The linearity of the standard curve was expressed in terms of the determination correlation coefficient (R^2) from the plots of the integrated peak area and concentration of the standard. According to the R^2 values (1.0000) of the calibration curves for each glucosinolate, the linearity of all calibration curves was acceptable. The limits of detection (LOD) and limit of quantification (LOQ) under the present chromatographic conditions were also determined with the formula (SD)*3; (SD)*10, respectively as shown in **Table 2**. In

addition, the recovery of the method was also evaluated by the analysis of all crude extract samples. All crude extracts showed values of recoveries of standard compounds ranged between of 101.52-111.36% and these values were acceptable.

Table 2. Retention time (t_R), LOD, and LOQ of glucosinolate standard compounds

Standard compounds	t_R (min)	LOD (µg/ml)	LOQ (µg/ml)	R^2
Progoitrin	8.87	0.03	0.09	1.0000
Glucotropaeolin	9.10	0.02	0.08	1.0000
Sinigrin	9.54	0.02	0.07	1.0000
Glucoraphanin	19.75	0.04	0.14	1.0000

The contents of the glucosinolate compounds in the unit of µg of glucosinolate compound/100 g of fresh weight (FW) in fresh green cabbage and fermented green cabbage products are shown in **Figure 5**.

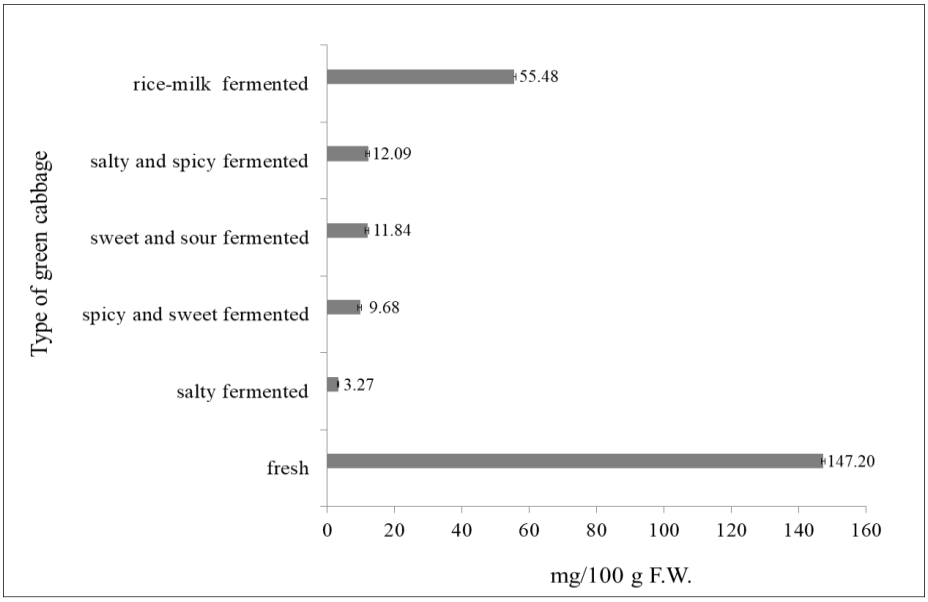

Figure 5. Quantitative analyses of glucosinolate compounds in the unit of mg /100 g of fresh weight (FW)

According to the data showed in **Figure 5**, it was found that only sinigrin was presented in fresh and fermented green cabbage products ranging from 3.27 to 147.20 mg/100 g FW and sinigrin was detected in fresh green cabbage with a highest amount. According to another report, sinigrin was major glucosinolate compound in brassica

vegetables especially green cabbage [Kushad M. M. *et.al.*, 1999; Verkerk R. *et.al.*, 2009]. After fermentation, all fermented green cabbage products showed the sinigrin content significantly lower than those from fresh green cabbage. It is worth to note that the fermentation condition such as pH, temperature, fermented duration and lactic acid formation may affect to glucosinolate content due to the decomposition of some glucosinolate compounds. In addition, some research have been reported that during the food processing of Brassica vegetables, the total GLS content decreases, three mechanisms being involved: (i) enzymatic breakdown involving myrosinase, a thioglucoside glycohydrolase (EC 3.2.3.1), (ii) leaking of GLS into the cooking water and (iii) thermal degradation [Usuki C. *et.al.*, 2006; Martinez-Villaluenda C. *et.al.*, 2009; Vicas S. I. et.al., 2015]. From the comparison of sinigrin content in all fermented products, it was found that the rice-milk fermented product exhibited the highest sinigrin content (55.48 mg/100 g of FW) while salty pickle fermented product exhibited the lowest level of sinigrin (3.27 mg/100 g of FW) . These results were corresponded to the study of total phenolic and flavonoid contents and antioxidant activity.

Conclusions

This work focused on the investigate the glucosinolate compounds and anti-nutritional factors including antioxidant activity, total phenolic acid and flavonoid contents from fresh green cabbage and four different fermented green cabbage products including salty fermented, spicy and sweet fermented, sweet and sour fermented, salty and spicy fermented and rice-milk fermented products. The result showed that, the fresh green cabbage showed the total phenolic and flavonoid contents, antioxidant activity and amount of glucosinolate compounds higher than those from fermented green cabbage products. The comparison of total phenolic and flavonoid contents and antioxidant activity in all fermented green cabbage products, it was found that the rice-milk fermented green cabbage product exhibited the highest amounts of phenolic and flavonoid contents and the best antioxidant activity as well. The amounts of glucosinolate compounds in fresh green cabbage and fermented green cabbage products were then analyzed by using HPLC technique. The method was validated and showed acceptable quantitative performance in terms of LOD, LOQ and accuracy. This could be applied to the identification and quantification of glucosinolate compounds in samples. The result showed that only sinigrin was presented in fresh and fermented green cabbage products in different amount. The highest sinigrin content was detected in fresh green cabbage while the fermented products resulted in significant reductions in amounts of sinigrin compounds. From the comparison of sinigrin contents in all fermented samples, it was found that rice-milk fermented green cabbage product exhibited the highest sinigrin content. This should be a good source of glucosinolates in fermented green cabbage product. Therefore, the data from this work might be greatly informative for the people who consume fresh green cabbage and fermented green cabbage products.

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