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REDUCTION OF GLUCOSINOLATES CONTENT DURING *SAYUR ASIN* FERMENTATION

[Penurunan Kandungan Berbagai Glukosinolat selama Fermentasi Sayur Asin]

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ABSTRACT

Glucosinolates (GLSs), health promoting compounds commonly found in *Brassica* vegetables, were studied during *sayur asin* fermentation made from Indian mustard (*B. juncea*). The current preliminary study aims to investigate the changes of glucosinolates content during 3 and 7 days of fermentation in two different media, *i.e.* coconut water and *tajin* liquor, and salt concentrations of 2.5 and 10%. The glucosinolates were analysed by HPLC after sample extraction in hot methanol followed by purification and de-sulphation. Results show that sinigrin was the most dominant glucosinolate among others, *i.e.* gluconapin, glucobrassicin, 4-hydroxy-glucobrassicin, 4-methoxy-glucobrassicin, and neo glucobrassicin, accounting for about 1000 and 4000 µmol/100 g dw in raw Indian mustard. Unfortunately, fermentation has substantially reduced the glucosinolates content in *sayur asin*. After 3 days of fermentation the sinigrin content was reduced by 95% as compared to that in the raw vegetable. The indole GLSs 4-methoxy-glucobrassicin and neo-glucobrassicin concentration decreased to 80-90% of the fresh materials. However, the decreasing mechanisms as well as factors contributing to the decrease of the glucosinolates could not be explained yet.

Keywords: fermentation, glucosinolates, health, *sayur asin*

ABSTRAK

Studi awal tentang kandungan glukosinolat, suatu kelompok senyawa fungsional yang biasa dijumpai di sayuran Brassica, selama fermentasi sayur asin dari sawi pahit (B. juncea) telah dilakukan dengan tujuan untuk mempelajari perubahan kandungan berbagai glukosinolat selama pembuatan sayur asin. Penelitian dilaksanakan dengan melakukan fermentasi sawi pahit selama tujuh hari dengan media air kelapa dan air tajin serta dua kadar garam (2.5 dan 10%). Glukosinolat diuji menggunakan HPLC setelah dilakukan ekstraksi, purifikasi, dan desulfatasi pada sampel. Hasil penelitian ini menunjukkan bahwa sinigrin merupakan glukosinolat yang paling dominan, berkisar 1000 dan 4000 µmol/100 g berat kering, di antara berbagai glukosinolat lain yang dapat diidentifikasi pada sawi pahit. Fermentasi telah menyebabkan turunnya kandungan berbagai glukosinolat secara drastis pada sayur asin dibandingkan pada sawi pahit segar. Pada hari ke-3 fermentasi, kandungan sinigrin turun hingga 95% dibanding pada sampel segar. Kandungan glukosinolat golongan indole (4-methoxy-glucobrassicin dan neo-glucobrassicin) juga menurun hingga 80-90% dibandingkan pada sawi pahit segar. Namun, mekanisme penurunan termasuk faktor-faktor yang mungkin berpengaruh belum dapat dijelaskan.

Kata kunci: *fermentasi, glukosinolat, kesehatan, sayur asin*

INTRODUCTION

Brassica vegetables, including cabbage, broccoli, and cauliflower, have been studied widely for the health promoting compounds, namely glucosinolates (GLSs). The GLSs are a group of β-thioglucoside N-hydroxysulphates with a sulphur linked β-D-glucopyranose moiety and side-chain group (R), which is derived mainly from one of certain amino acids, such as methionine, tryptophan, and phenylalanine. The GLS can be classified either as aliphatic, aromatic, or indole (Clarke, 2010; Verkerk *et al.* 2009; Halkier and Gershenzon, 2006). Upon damage of plant tissue, GLSs are highly prone to hydrolysis catalysed by myrosinase enzyme (β-thioglycosidase EC 3.2.1.147) (Bones and Rossiter, 2006; Verkerk *et al.* 2009). The GLSs are believed to lower the risk of cancer, particularly due to isothiocyanates, one of the GLSs derivatives, which can inhibit phase 1 and induce phase 2 enzymes during carcinogen metabolism (Traka and Mithen, 2009). Reviews of epidemiological studies reported inverse associations between intake of *Brassica* vegetables and the risk of certain cancers (Cartea and Velasco, 2008; Herr and Büchler, 2010).

Fermentation is one of the methods to prepare *Brassica* vegetables, *e.g.* sauerkraut (Ciska and Pathak, 2004; Uva *et al.* 2006). However, fermentation commonly leads to GLSs loss in the product (Daxenbichler *et al.* 1980; Ciska and Pathak, 2004; Martinez-Villaluenga *et al.* 2009). To our knowledge, there is no study on the GLSs behaviour along the *sayur asin* production. *Sayur asin* is prepared by spontaneous fermentation of Indian mustard in the presence of salt. Either *tajin* liquor or coconut water is used as the medium. This paper aims to describe the changes of GLSs content in Indian mustard during *sayur asin* production and how these are affected by the fermentation medium and salt concentration.

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MATERIALS AND METHODS

Preparation of *sayur asin*

Raw Indian mustard (*Brassica juncea*), local name: *sawi pahit*, was obtained from a local supplier in Bandungan, Central Java. Sampling was performed from the pooled vegetables after randomly divided them into number of treatments and replication. Each treatment contained approximately 1500 grams of Indian mustard or six whole plants for three replications. Each vegetable was washed by running tap water followed by draining. Subsequently it was withered under sunlight for four hours. Each vegetable was added with salt (2.5 or 10% w/w) and rubbed gently by hand simultaneously. Subsequently, it was immersed into a glass jar containing either the *tajin* liquor (1:2 w/v) (Nugerahani *et al.* 2000) or coconut water (1:5 w/v). The ratio was estimated after interviewing two local *sayur asin* producers. Eventually each sample was incubated at 25 - 30°C for 7 days. Each jar represented a replication of one treatment, containing two whole plants. Therefore, there were six samples per treatment for glucosinolates analysis, except for the samples of raw vegetable (n = 4) and coconut water-10% salt-day 3 (n = 5).

Tajin liquor was prepared by boiling rice (IR-64) in distilled water at a ratio 1:14 (w/v) for 15 min. Subsequently, salt was added at a ratio 1:40 (w/v of added water) and mixed. The liquid was separated and cooled at room temperature. Fresh coconut water was obtained from the local market. The glucosinolates were analysed in raw vegetable and after three and seven days of fermentation based on common practice of local producers. Subsequent experiment was performed to study glucosinolates during a shorter fermentation period, *i.e*. three days. The withered vegetable was fermented in coconut water (1:5 w/v) and 2.5% (w/w) of salt. The glucosinolates were analysed in raw and withered vegetables, one and three hours after immersion into the medium, and one to three days of fermentation. Each treatment was represented by six samples for analysis

Sample preparation

Both raw and processed vegetables were chopped into pieces of approximately less than 1 cm and mixed thoroughly. The chopped vegetable was frozen in liquid nitrogen followed by grinding in a Waring blender and stored in boxes at -20°C until further lyophilisation (ThermoScientific LL150). Samples of fermentation media were stored in freezer at -20°C.

Extraction of glucosinolate

The extraction of glucosinolates (GLSs) was employed twice in each replication and used hot methanol (Merck) as described by Verkerk *et al.* (2001) with minor modifications. Lyophilised sample of 0.1 g was added with 2.4 mL hot methanol 70% and 1 mL glucotropaeolin 3 mM (provided by Food Quality and Design, WU) was added as the internal standard. Sample was incubated in waterbath at 75°C for 20 minutes and it was mixed every 5 minutes. Then, sample was centrifuged for 10 min at 6000 rpm (Hettich EBA20). The supernatant was collected while residue was re-extracted twice with 10 mL of hot methanol following similar procedure. Eventually all supernatants were combined.

Purification and de-sulphation

The extracted GLSs were purified and de-sulphated on a 1.5 cm DEAE Sephadex A-25 (Sigma-Aldrich) anion exchange in glass-wool (Merck) matrix column. The column was washed twice with 1 mL bi-distilled water, and loaded with 2 mL of the GLSs extract and washed twice with 1 mL sodium acetate (Merck) solution 20 mM. Sulphatase (Sigma-Aldrich) was added and it was incubated overnight at room temperature (23–27°C). Then, the de-sulpho-GLSs were eluted three times with 0.5 mL bi-distilled water. Finally, the elute was filtered by a 0.45 µm filter prior to HPLC analysis.

HPLC analysis

HPLC set-up used LC 10 Avp system (Shimadzu) comprising a pump system with low pressure gradient valve, a degasser, a system controller, and a converter board. A UV Vis detector was performed at a wavelength of 229 nm. Results were analysed by using Class VP software. The de-sulpho-GLSs were separated by GraceSmart RP-18 5µ column at 1 mL/min flow rate. The elution was a gradient system of water containing 0.05% tetramethyl-ammonium chloride (code *A*) and acetonitrile (Merck)/water (20:80, v/v) containing 0.05% tetramethyl-ammonium chloride (code *B*). Total elution time was 31 min and gradient as follows: 100% A for 1 min, then within 20 min to 100% B and within 5 min back to 100% A followed by maintaining 100% A for 5 min. The GLSs were identified by comparing with standards of sinigrin and glucotropaeolin (provided by Food Quality and Design WU), and known GLS profiles of broccoli, cauliflower, red cabbage, radish, and *Brussels* sprouts, and confirming literatures. Each GLS was quantified as response factor against glucotropaeolin as internal standard. Values of glucosinolates will be presented in table and figure as mean ± SEM.

RESULTS AND DISCUSSION

Glucosinolates in Indian mustard

The glucosinolates (GLSs) identified in Indian mustard were aliphatics, *i.e.* sinigrin and gluconapin, and indoles, *i.e.* glucobrassicin, 4-hydroxy-glucobrassicin, and 4-methoxy-glucobrassicin and neo glucobrassicin, as shown in the chromatogram (Figure 1). Moreover, the GLS sinigrin accounted for more than 90% of the total GLSs. In general, the GLSs identified are in accordance with previous reports (Font *et al.* 2004; He *et al.* 2003[a]). However, He *et al.* (2003[a]) reported that gluconapin and progoitrin were dominant GLSs in growing potherb mustard (*B. juncea* Coss). Discrepancies in the GLS profile could be due to the differences in variety, agricultural conditions, and the analytical methods.

Effect of fermentation on the glucosinolates content

Fermentation substantially reduced the GLSs content in Indian mustard during fermentation (Table 1).

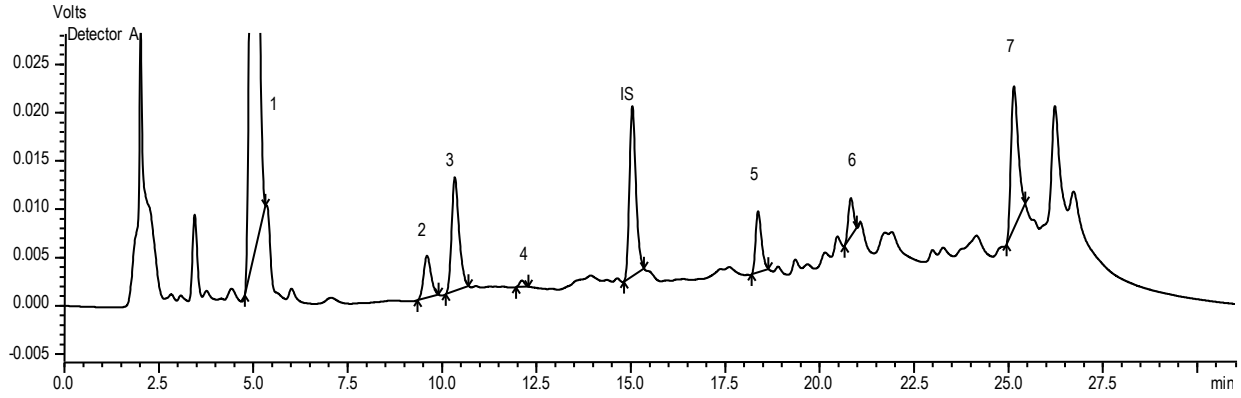

Figure 1. An example of chromatogram of glucosinolates and {the *Rt*} in Indian mustard: (1) Sinigrin {5.29}, (2) Gluconapin {9.99}, (3) Unknown {10.63}, (4) 4-Hydroxy-glucobrassicin (4-OH-GBR) {12.40}, (IS) Glucotropaeolin {15.25}, (5) Glucobrassicin {18.51}, (6) 4-Methoxy-glucobrassicin (4-Me-GBR) {20.95}, and (7) Neo-glucobrassicin (Neo-GBR) {25.24}

At the third fermentation day, there was a remarkable drop of sinigrin for more than 95% compared to sinigrin in raw vegetable. Meanwhile, the concentration of indole 4-methoxy-glucobrassicin and neo-glucobrassicin decreased at 80-90% as compared to the ones in raw vegetable. Subsequent experiment was performed in a shorter period of fermentation. Sinigrin, as the most dominant GLS among others in the Indian mustard, is chosen to describe the GLS changes during fermentation (Figure 2). This study shows that initial sinigrin content in Indian mustard was about 4 times lower compared to the one in the first experiment. It is likely that biological variability of the vegetable may affect GLS concentrations (He *et al.* 2003b; Krumbein *et al.* 2005; Verkerk *et al.* 2009). Interestingly, sinigrin content slightly increased after withering until three hours of the vegetable immersion into medium. Previous studies also reported an increase of GLSs level, *e.g.* after microwave processing of red cabbage (Verkerk and Dekker, 2004), boiling of cauliflower (D'Antuono *et al.* 2007), and steaming of broccoli (Gliszczyñska-šwiglo *et al.* 2006; Miglio *et al.* 2008). It has been shown previously that such treatments (in this case are withering and fermentation) might increase the extractability of GLS from the sample matrix in analytical methods. Afterward, sinigrin of the day 1 sample decreased drastically at about 80% as compared to the previous hour 3 sample. Subsequent decrease was observed at the day 2 and 3 of fermentation resulting in 7-8% of sinigrin in raw vegetable. Previous studies reported that fermentation significantly reduce GLSs in *Brassica* vegetables. During sauerkraut production, Tolonen *et al.* (2002) observed a very low quantity of 4-methoxy-glucobrassicin while, others reported no GLSs in fermented product (Daxenbichler *et al.* 1980; Martinez-Villaluenga *et al.* 2009). Meanwhile, Suzuki *et al.* (2006) reported smaller quantities of the GLSs in *nozawana-zuke*, a fermented product of *Brassica rapa* L. in Japan, including glucobrassicin and gluconasturtiin as the major GLSs, relative to the fresh *nozawana*. The most recent study on white cabbage fermentation reported that GLSs content can be retained by a thermal treatment prior to fermentation. After 71 h of fermentation the cabbage still contained 35% of total GLSs before fermentation (Sarvan *et al.* 2013).

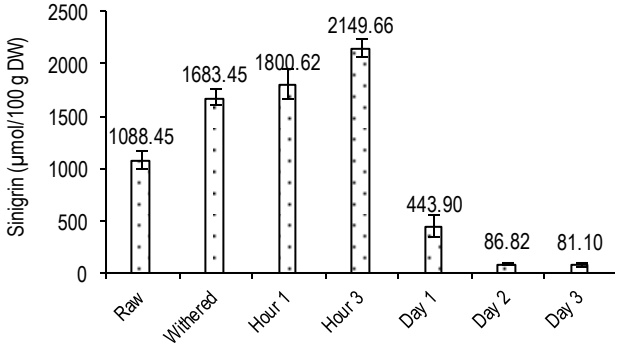

Figure 2. Sinigrin content during *sayur asin* production in coconut water (1:5, w/v) and 2.5% (w/w) of salt. Values show mean ± SEM ($n = 6$)

The contents of GLS derived products depend on the fermentation conditions as well as the concentrations of GLS of the raw material (Ciska and Pathak, 2004). Previous studies reported GLS changes during fermentation based on the changes of identified breakdown products, *e.g.* isothiocyanates (ITCs) and cyanides from aliphatic GLSs, indole-3-carbinol and ascorbigen from glucobrassicin, and 2-phenylethyl ITCs from gluconasturtiin (Ciska and Pathak, 2004; Daxenbichler, 1980).

In the context of significant drops of GLSs content, the current result shows that possible effects of fermentation medium and salt concentration could not be explained clearly. In general, the use of *tajin* liquor may affect to the lower sinigrin than the one using coconut water. While, higher salt concentration (*i.e.* 10%) may also affect to the lower sinigrin retention at the third day of fermentation even though, this was not clearly indicated in the *sayur asin* in coconut water. Further studies are needed to confirm the finding and to see any interactions between variables influencing GLSs content.

Mechanisms affecting GLSs content during fermentation are different from other processing by applying heat; however, these are scarcely studied (Nugrahedi *et al.* 2013). Bacteria and sodium chloride might take significant role to change the GLSs profile (Tolonen *et al.* 2002; Suzuki *et al.* 2006; Martinez-Villaluenga *et al.* 2009). While, leaching of GLSs into the medium is scarcely involved in the *sayur asin*. Accordingly, Suzuki *et al.* (2006) reported the GLSs retention in the tissues after fermentation.

Table 1. Glucosinolates in *sayur asin* production (µmol/100 g dw)

	Sinigrin	Gluconapin	4-OH-GBR	Glucobrassicin	4-Me-GBR	Neo-GBR
Raw	4216.5 ± 118.3	189.3 ± 10.7	5.2 ± 0.4	68.4 ± 13.9	30.9 ± 1.8	106.6 ± 8.6
C,2.5%,3d*	53.9 ± 12.7	ND	ND	ND	4.1 ± 0.6	21.9 ± 2.6
C,2.5%,7d	33.2 ± 12.5	ND	ND	ND	2.5 ± 0.7	4.6 ± 2.0
C,10%,3d	48.1 ± 6.3	ND	ND	ND	4.5 ± 0.8	23.0 ± 2.8
C,10%,7d	52.6 ± 10.0	ND	ND	ND	6.5 ± 1.1	22.2 ± 5.4
T,2.5%,3d	54.6 ± 12.5	ND	ND	ND	5.8 ± 1.0	12.9 ± 6.2
T,2.5%,7d	16.9 ± 1.8	ND	ND	ND	4.6 ± 1.0	20.7 ± 4.5
T,10%,3d	23.7 ± 2.5	ND	ND	ND	4.8 ± 1.5	16.1 ± 4.4
T,10%,7d	31.4 ± 9.6	ND	ND	ND	4.9 ± 1.2	16.2 ± 2.6

Values show mean ± SEM. *Sequence of treatment code: medium (C: coconut water; T: *tajin*), salt concentration, days of fermentation; ND – Not Detectable

CONCLUSION

Sinigrin was the most dominant glucosinolate among others, *i.e.* gluconapin, glucobrassicin, 4-hydroxy-glucobrassicin, 4-methoxy-glucobrassicin, and neo glucobrassicin, accounting for about 1000 and 4000 µmol/100 g dw in raw Indian mustard. However, fermentation has reduced the glucosinolates content in *sayur asin*. At the third day of fermentation, there was a substantial drop of sinigrin for more than 95% compared to raw vegetable. There is no clear mechanism of factors contributing the reduction. Further studies are needed to identify the factors, *e.g.* pH, salinity, substrate, lactic acid bacteria, as well as myrosinase activity, and explain the working mechanisms underlying the glucosinolates degradation.

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