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Behavior of Glucosinolates in Pickling Cruciferous Vegetables

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Crucifer species, which include widely consumed vegetables, contain glucosinolates as secondary metabolites. Cruciferous vegetables are consumed in Japan in salt-preserved or pickled form as well as cooked and raw fresh vegetables. In this study, changes in contents of glucosinolates during the pickling process were investigated. 4-Methylthio-3-butenyl glucosinolate, a major glucosinolate in the root of Japanese radish, daikon (*Raphanus sativus* L.), was detected in pickled products with a short maturation period but not in those with a long maturation period. As a model pickling experiment, fresh watercress (*Nasturtium officinale*) and blanched watercress were soaked in 3% NaCl solution for 7 days. The results showed that the ratio of indole glucosinolates to total glucosinolates increased during the pickling process, whereas total glucosinolates decreased. Myrosinase digestion of glucosinolates in nozawana (*Brassica rapa* L.) indicated that indole glucosinolates, especially 4-methoxyglucobrassicin, were relatively resistant to the enzyme. The effect of pickling on glucosinolate content and the possible mechanism are discussed in view of degradation by myrosinase and synthetic reaction in response to salt stress or compression during the pickling process.

KEYWORDS: Glucosinolate; myrosinase; pickling; *Brassica rapa* L.; *Nasturtium officinale*

INTRODUCTION

Glucosinolates are the major secondary metabolites in many crucifer species and are also found in noncruciferous dicotyledonous species. Glucosinolates are β-thioglucoside *N*-hydroxyiminosulfate esters, which have a sulfur-linked β-D-glucopyranose moiety and a side chain on an α carbon of the imino group (**Figure 1**). Glucosinolates are classified into three groups by their side-chain structures: aliphatic, indole, and aromatic glucosinolates derived from methionine, tryptophan, and phenylalanine or tyrosine, respectively. So far, more than 120 different glucosinolates have been identified (*1*). Glucosinolates are stored in vacuoles and decomposed by cytosolic and apoplastic myrosinase/thioglucosidase (EC 3.2.3.1) upon tissue damage into a variety of volatile and nonvolatile bioactive compounds, the characteristic flavor and taste of which distinguish cruciferous vegetables from other vegetables. It has been demonstrated that some specific hydrolysis products such as isothiocyanates and indole-3-carbinol are capable of modulating biotransformation enzyme activity and preventing certain cancers (*2*, *3*). A high dietary intake of cruciferous vegetables has been consistently associated with protection against a range of cancers (*4*, *5*).

The glucosinolate content of fresh vegetables is considerably affected by many factors such as soil type, plant spacing, light, temperature, and application of sulfate and nitrogenous fertilizer. Furthermore, the concentrations and types of glucosinolates vary greatly with plant variety and species, tissue type (roots, leaves, flowers, seeds, etc.), and the developmental stages of the tissue (*6*, *7*). In daikon root, for example, the glucosinolate content in the phloem of the root tip is much higher than that in the upper part of the root (*8*).

Cruciferous vegetables account for 30% of all vegetables marketed in Japan according to the Statistics of Agriculture, Forestry and Fisheries, and many types of traditional cooking and pickling, which vary according to locality, have been developed. One of the most popular and important cruciferous crops in Japan is daikon, a Japanese radish (*Raphanus sativus* L.), which has the highest levels of production and consumption among vegetables grown in Japan. A wide variety of cooking and pickling techniques for this vegetable exist, with many local variations in pickling processes. The pungent flavor of daikon products is mainly derived from a degradation product of 4-methylthio-3-butenyl glucosinolate (dehydroerucin), 4-methylthio-3-butenyl isothiocyanate. This product is further converted to a yellow pigment that distinguishes pickled products such as takuan-zuke (*9*, *10*). The total glucosinolate content of daikon was reported as 259 ± 55 μmol/100 g of fresh weight, and the mean dehydroerucin content corresponded to about 80% of that of total glucosinolates (*8*). On the other hand, there are various regional cruciferous crops with limited usage. For example, the leaves of nozawana (*Brassica rapa* L.) are mostly processed to pickled products as local specialties of the Nagano area, and

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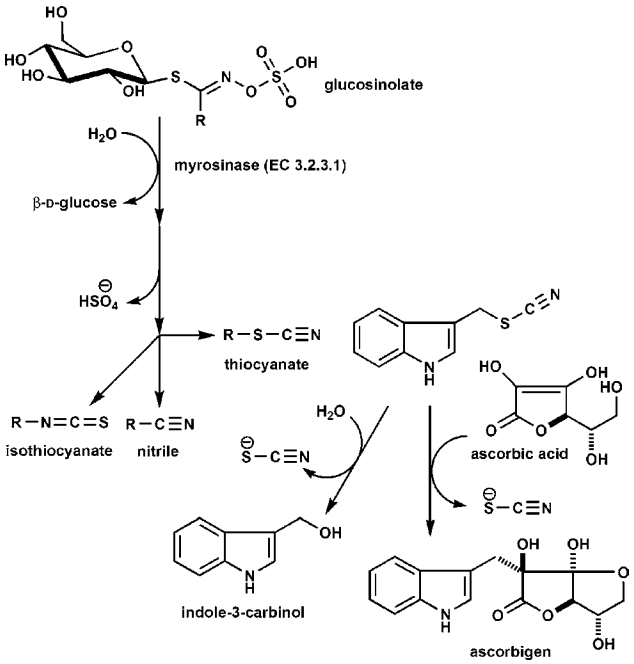

Figure 1. Degradation and conversion of glucosinolates. Glucosinolates are hydrolyzed by the endogenous enzyme myrosinase to form glucosinolate hydrolysis products: nitriles, isothiocyanates, and thiocyanates. Indol-3-ylglucosinolate (glucobrassicin) is further converted to bioactive products, such as indole-3-carbinol and ascorbigen.

fresh leaves are not shipped to the market. Some pickled forms of this vegetable are produced in China.

Although cruciferous vegetables have been studied widely because of their beneficial effects on health, the effect of processing on their bioactive compounds is still not well investigated (*11*). Previous study of glucosinolates and their degradation products during fermentation has been limited to fermented cabbage (sauerkraut) (*12*, *13*). Despite the variety of cruciferous vegetables and the wide range of processing methods in Japan, limited analysis of the processed vegetables has been conducted. As a high dietary intake of cruciferous vegetables is thought to be desirable for preventing certain cancers, development of methods for retaining glucosinolates and their functional degradation products is expected to aid in the production of cruciferous vegetable products with greater health benefits. In this study, the distribution and behavior of glucosinolates in cruciferous vegetables during the pickling process were investigated to obtain information on the metabolism and degradation of glucosinolates.

MATERIALS AND METHODS

Chemicals and Reagents. Sinigrin monohydrate, myrosinase (from *Sinapis alba*), and sulfatase (EC 3.1.6.1) (type H-1) were obtained from Sigma (St. Louis, MO). HPLC grade methanol and trifluoroacetic acid were purchased from Nacalai Tesque (Kyoto, Japan).

Samples and Sample Preparation. Fresh daikon roots and various pickled daikon products were purchased from supermarkets in Japan. Daikon kimchi is a traditional fermented Korean food prepared through a series of processes including brining and blending of daikon with various spices and other ingredients. Benibana daikon is a local specialty of the Yamagata area, which is pickled with sweetened vinegar and petals of safflower (benibana, *Carthamus tinctorius* L.). Takuan-zuke is made of dried daikon with rice bran and salt. For bettara-zuke, daikon is brined and then pickled with malted rice, which adds a sweet taste to the product. Miso-zuke is made of brined daikon preserved in miso paste until it has the dark color of miso.

Rocket salad (*Eruca vesicaria* ssp. *sativa*), kaiware (*Raphanus sativus* sprouts), and broccoli sprouts (*Brassica oleracea* L.) were purchased from supermarkets in Japan. Fresh watercress (*Nasturtium officinale*) from distinct production areas (Tochigi and Gunma) was purchased from supermarkets and was also obtained from a farm in Ibaraki. Fresh nozawana leaves and its pickled product (nozawana-zuke) and fresh seisai (*Brassica juncea* Czern. et Coss.) were obtained from farms in Nagano and Yamagata, respectively. Samples were pulverized in liquid N_2 using a mortar and pestle and freeze-dried.

Enzyme Treatments. To examine the myrosinase sensitivity of HPLC-purified glucosinolates, each lyophilized glucosinolate sample (approximately 3−5 μg) was dissolved in 100 μL of 25 mmol/L phosphate buffer (pH 6.5) containing 0.5 mmol/L ascorbic acid and 0.5 mg of myrosinase (Sigma) and incubated for 30 min at 30 °C. After the addition of 2 μL of 5% TFA, the reaction mixture was applied to Ultrafree-MC 5000 NMWL, and the filtrate was analyzed by HPLC.

To obtain desulfoglucosinolates, lyophilized glucosinolate samples were applied to a Sep-Pak Plus QMA cartridge equilibrated with water. The cartridge was washed with 5 mL of water, and 0.3 mL of 0.2% sulfatase was applied. After 2 h of incubation at ambient temperature, desulfoglucosinolates were eluted with 0.7 mL of water. The remaining glucosinolates were eluted with 0.5 mL of 0.3 mol/L ammonium sulfate to estimate the efficiency of the enzyme reaction. These samples were analyzed by HPLC, according to the method of Kiddle et al. (*14*).

Table 1. Identification of Glucosinolates in Watercress and Nozawana

				$[M-H]^-$ (*m/z*)	
peak[a]	RI (min)	glucosinolate side chain	common name (abbreviation)	obsd	calcd[b]
Watercress					
2	16.0	6-(methylsulfinyl)hexyl	glucoheperin (GHp)	464.0730	464.0724
3	16.7	4-hydroxyindol-3-ylmethyl	4-hydroxyglucobrassicin (4HGBr)	463.0492	463.0487
4	17.5	(*R*)-2-hydroxy-2-phenethyl	glucobarbarin (GBb)	435.0540	438.0534
5	18.5	(*S*)-2-hydroxy-2-phenethyl	glucosibarin (GSi)	435.0539	438.0534
6	19.3	7-(methylsulfinyl)heptyl	glucosiberin (GSb)	478.0884	478.0881
8	21.3	indol-3-ylmethyl	glucobrassicin (GBr)	447.0545	447.0537
9	23.5	2-phenylethyl	gluconasturtiin (GNs)	422.0591	422.0585
10	23.9	8-(methylsulfinyl)octyl	glucohirsutin (GHr)	492.1041	492.1037
12	25.8	4-methoxyindol-3-ylmethyl	4-methoxyglucobrassicin (4MGBr)	477.0644	477.0643
Nozawana					
1	13.7	3-butenyl	gluconapin (GNp)	372.0459	372.0428
3	16.9	4-hydroxyindol-3-ylmethyl	4-hydroxyglucobrassicin (4HGBr)	463.0484	463.0487
7	19.3	4-(methylthio)butyl	glucoerucin (GEr)	420.0462	420.0462
8	21.5	indol-3-ylmethyl	glucobrassicin (GBr)	447.0534	447.0537
9	23.9	2-phenylethyl	gluconasturtiin (GNs)	422.0585	422.0585
11	24.0	5-(methylthio)pentyl	glucoberteroin (GBt)	434.0616	434.0619
12	26.1	4-methoxyindol-3-ylmethyl	4-methoxyglucobrassicin (4MGBr)	477.0640	477.0643

[a] Peak numbers correspond to the numbers in **Figure 2**. [b] Exact masses were calculated on the basis of the atomic weights reported in 2003 (*35*).

Simulation of Pickling. Fresh watercress was washed and divided into two experimental groups. In experiment 1, watercress (100 g) was directly soaked in 300 mL of 3% NaCl, pressed in a household pickling container with a spring, and kept at 4 °C for 7 days. In experiment 2, watercress (100 g) was quickly blanched and soaked in 300 mL of 3% NaCl as in experiment 1. Blanching was performed by pouring boiling water over the vegetables in a sieve basket. Approximately 6−10 g of salted materials was sampled each day and pulverized in liquid N_2, as described above.

Glucosinolate Analysis. Glucosinolates were extracted from 50 mg of the freeze-dried powder and 0.2 mL of 1 mg/mL sinigrin as an internal standard with 2 mL of boiling water for 15 min. After centrifugation (12000*g*, 10 min at 4 °C), supernatants were applied to an Ultrafree-MC 5000 NMWL ultrafiltration filter unit (Millipore, Bedford, MA). The filtrate was analyzed by HPLC.

A Shimadzu LC10AD HPLC system with an SPD-M10A*vp* photodiode array detector (Shimadzu, Kyoto, Japan) was used for all HPLC analyses. The photodiode array detector was set to scan between 200 and 360 nm, and the response at 235 nm was plotted. For the detection of intact glucosinolates, 250 × 4.6 mm i.d., 5 μm, and 100 × 4.6 mm i.d., 3 μm, Capcell Pak C18 AQ reverse-phase columns (Shiseido, Tokyo, Japan) were used in conventional analysis and analysis of myrosinase digestion, respectively, at ambient temperature with a flow rate of 0.8 mL/min. A 250 × 10 mm i.d., 5 μm, Capcell Pak C18 AQ column was used for large-scale sample preparation for NMR analysis with a flow rate of 2 mL/min. The mobile phase used was a linear gradient from 100% dolvent A [water containing 0.05% trifluoroacetic acid (TFA)] to 100% solvent B (80% methanol containing 0.05% TFA) typically in 40 min (*15*). To analyze desulfoglucosinolates, a 250 × 4.6 mm i.d., 5 μm, Capcell Pak C18 AQ column with a flow rate of 1 mL/min was used. The mobile phase used was a linear gradient from 100% water to 40% acetonitrile, according to the method of Kiddle et al. (*14*).

The amount of glucosinolates was estimated using absorbance of 235 nm compared with sinigrin as the standard.

Statistical Analysis. Statistical analysis was carried out according to the General Linear Model (GLM) procedure using the SAS system (version 9.1, SAS Institute, Cary, NC) with Dunnett's test ('DUNNETTL' option).

Mass Spectrometry. LC-MS/MS analyses were performed on an API 4000 Qtrap mass spectrometer (Applied Biosystems, Foster City, CA) coupled with an HP 1100 HPLC system (Agilent, Palo Alto, CA). A 100 × 2 mm i.d., 3 μm, Capcell Pak C18 AQ column (Shiseido) was used for all LC-MS/MS analyses. Glucosinolates were eluted with a linear gradient, increasing the ratio of solvent B (90% methanol containing 0.1% formic acid) against solvent A (1% methanol containing 0.1% formic acid) from 0 to 100% in 25 min, after which 100% B was maintained for 10 min and the column was equilibrated with 100% A for 15 min. Column chromatography was carried out at 40 °C with a flow rate of 0.2 mL/min. After 3 min of each sample injection, the eluate was transferred to the mass spectrometer, which was operated in the negative-ion mode. The electrospray ionization (ESI) parameters and triple-quadrupole parameters were optimized using sinigrin as follows: ion spray voltage, −4.5 kV; nebulizer gas (GS1), 40 psi; turbo gas flow (GS2), 80 psi; curtain gas, 20; heated-nebulizer temperature, 600 °C; collisionally activated dissociation (CAD), 10; declustering potential (DP), −60 to −85 V; entrance potential (EP), −10; collision energy (CE), −36 V; collision cell exit potential (CXP), −15 V. The scan ranges for precursor ion scans and product ion scans were *m*/*z* 200−600 and 50−600, respectively. The data obtained were processed using Analyst 1.4 software (Applied Biosystems).

Accurate mass analyses were performed on an ApexII (7 T) Fourier-transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA). After fractionation by HPLC, the fractions were diluted with 50% methanol in 3% acetic acid and infused by a syringe pump through the Analytica ESI source at a flow rate of 0.1 mL/h. The cell conditions were tuned by sinigrin to achieve a spectrum of good intensity. The mass spectrometer was calibrated with dihydroxybenzoic acid, gallic acid, adenosine monophosphate, and the dimer in the negative-ion mode. Spectra were recorded at a resolution of approximately 50000 in the mass range of the $[M - H]^-$ value ± 6 units using 8K data points. The elemental composition was determined among candidates with mass errors of <2 ppm, which were calculated using the software XMASS 5.0 (Bruker Daltonics).

NMR Spectroscopy. To isolate glucosinolate samples for NMR analysis, 1 g of freeze-dried, powdered nozawana leaves was suspended in 50 mL of boiling water and kept boiling for 15 min. After homogenization with an Ultra-Turrax T25 homogenizer (IKA Japan), the samples were centrifuged twice (10000*g*, 15 min at 4 °C) to precipitate the insoluble material. Large molecules such as proteins in the supernatant were removed by ultrafiltration using a Centriprep-3 (Millipore). Five milliliters of the filtrate was applied to a Sep-Pak Plus QMA cartridge equilibrated with water. The cartridge was washed with 10 mL of water, and glucosinolates were eluted with 1 mL of 0.3 mol/L ammonium sulfate. The glucosinolate fraction was applied to HPLC.

An elution peak corresponding to the unidentified glucosinolate was collected and lyophilized. The lyophilized sample (165 μg) was dissolved in D_2O. ^1H NMR spectra were obtained at 800.02 MHz on a Bruker Avance 800 (Bruker Biospin, Karlsruhe, Germany) at 298 K. The ^{13}C NMR spectrum was obtained at 125.77 MHz on a Bruker Avance 500 (Bruker Biospin) at 298 K.

RESULTS AND DISCUSSION

Analysis of Dehydroerucin in Pickled Products of Daikon. Daikon is one of the most popular and important cruciferous vegetables in Japan. However, information on the chemical components of pickled daikon products is limited in spite of the wide variety of pickled products available. Dehydroerucin, also called glucoraphasatin, is the most abundant glucosinolate in daikon, and its degradation product, 4-methylthio-3-butenyl isothiocyanate, has been shown to have antimutagenic activity (*16*) and reducing capacity (*17*). We examined the content of dehydroerucin in various pickled daikon products. Among the samples analyzed, the contents of dehydroerucin in benibana-daikon, kimchi, and bettara-zuke were 54.2 ± 5.6, 29.4 ± 0.2, and 24.3 ± 5.1 μmol/100 g of fresh weight, respectively. These products were produced with a relatively short pickling period (1−2 weeks). On the other hand, takuan-zuke and miso-zuke, which require a long maturation period (1−3 months), did not retain dehydroerucin and therefore would not serve as important dietary sources of this substance.

Dehydroerucin in daikon may be degraded by endogenous myrosinase during the pickling process, resulting in the volatile degradation product, 4-methylthio-3-butenyl isothiocyanate, which is further converted to a yellow pigment (*9*, *10*). To evaluate the myrosinase activity in the brining process, we examined in vitro myrosinase activity against sinigrin in the presence of various concentrations of NaCl (10−500 mM) at various pH values (4−7.5). No activity was observed in the presence of 500 mM NaCl and below pH 5.5, which corresponds to the brining condition with high salt concentration and low pH level. Therefore, the period when myrosinase could digest glucosinolates may be limited. The timing when brining NaCl reaches the daikon tissue cells depends on the cutting size, and myrosinase could not react with the substrates until NaCl distributes throughout the tissues. This may be the why dehydroerucin remained in the pickled products having short maturation periods. Besides, the long-maturation pickles, such as takuan-zuke, were pickled after being partially dried. In the drying process, the membrane structure of the tissues may be disrupted, resulting in easy contact of myrosinase with the substrate.

Ingredients or materials used for pickling, such as rice bran, malted rice, and miso, and the ingredients of kimchi, are rich in enzymes or lactic acid bacteria. Malted rice, which contains enzymes of koji mold (*Aspergillus oryzae*), is used for bettara-

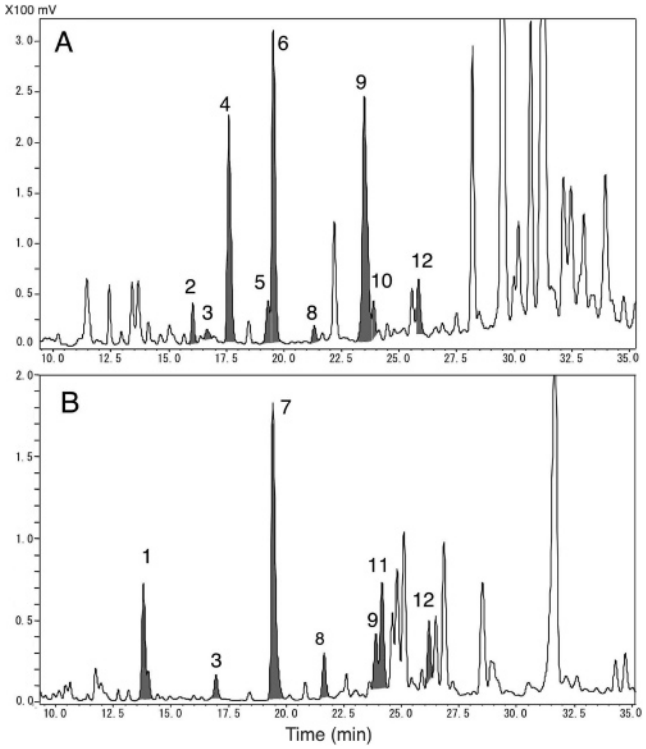

Figure 2. HPLC profiles of intact glucosinolates derived from (**A**) fresh watercress and (**B**) fresh nozawana. Peaks that contained glucosinolates listed in **Table 1** are highlighted gray. Peak numbers refer to the glucosinolates listed in **Table 1**.

zuke. Miso is fermented soybean paste using enzymes of koji. Fermented rice bran or kimchi is rich in lactic acid bacteria. The contribution of such enzymes and microorganisms to the degradation of dehydroerucin cannot be ruled out. Glucosinolate degradation activities of bacterial strains derived from human intestinal microflora (*18*), such as Bacteroides (*19*) and *Bifidobacterium* sp. (*20*), and strains obtained by screening, such as *Lactobacillus agilis* (*21*), have been reported, whereas myrosinase genes have not been found in microorganisms despite recent progress of genome projects. Enzymes other than myrosinase may, therefore, contribute to the bacterial degradation of glucosinolates. In this study, the degradation product of dehydroerucin, 4-methylthio-3-butenyl isothiocyanate, was not analyzed. Therefore, the effect of such ingredients and bacteria on the degradation product should be investigated further.

Identification of Glucosinolates in Watercress and Nozawana. To examine the behavior of glucosinolates during pickling, we performed a model experiment to simulate the pickling process. To select model vegetables that would be good sources of glucosinolates, we examined the glucosinolate content in various vegetables, including rocket salad, broccoli sprouts, watercress, seisai, kaiware, daikon root, and nozawana (data not shown). The elution peaks of glucosinolates and their side-chain types were estimated from absorbance spectra monitored by photodiode array (*14*). The presence of a peak at around 225 nm in the UV spectrum is characteristic of glucosinolates, and absorption between 250 and 290 nm and a shoulder at around 230 nm are indicative of indole glucosinolates and aromatic glucosinolates. Among the vegetables we analyzed, watercress, nozawana, and broccoli sprouts contained more than six glucosinolates belonging to various groups.

Glucosinolates in each elution peak of fresh watercress (**Figure 2A**) and nozawana (**Figure 2B**) were identified by LC-MS/MS and confirmed by accurate mass analyses (**Table 1**). Each mass of $[M - H]^-$ was in agreement with the mass of the corresponding glucosinolate. Glucosinolates with the same molecular masses were distinguished by their retention times, as previously described (*22*). The component of elution peak 12 was estimated to be 4-methoxyglucobrassicin or neoglucobrassicin from the molecular mass. As intact forms of these glucosinolates had the same retention time (*22*), the glucosinolate isolated from peak 12 was treated with sulfatase and analyzed by HPLC. The retention time of the desulfoglucosinolate derived from peak 12 was 23.2 min, suggesting that the glucosinolate was 4-methoxyglucobrassicin, not neoglucobrassicin (*14*). Elution peak 1 contained a glucosinolate with a molecular mass of 373, which could not be identified by LC-MS/MS. It was analyzed by NMR and identified as gluconapin (3-butenyl glucosinolate) by the presence of 4-pentenimine and glucose moieties, the structures and connection of which were determined by DQF-COSY and HMBC spectra (data not shown).

Behavior of Glucosinolates in the Simulated Pickling Process of Watercress. Although a wide variety of pickling processes for cruciferous vegetables are used in Japan, studies on the behavior of glucosinolates during the pickling process have been limited. In particular, no published studies have examined the relationship between side-chain structure and degradation rates of glucosinolates. To examine the behavior of each glucosinolate during the pickling process, we selected watercress as a model vegetable. In most cases, the first step of pickling is salting vegetables in 3−4% NaCl. As a simulation of the pickling process, fresh watercress was divided into two experimental groups. Fresh watercress (experiment 1) or watercress blanched by scalding with boiling water (experiment 2) was soaked in 3% NaCl. Blanching slows or stops the action of enzymes so that texture, color, and flavor are preserved and is sometimes used for leaf vegetables before pickling. The decreases in the intensities of glucosinolate peaks were monitored, as shown in **Figure 3**.

In experiment 1, the mean value of each glucosinolate decreased after 7 days of pickling, although the decreases were not statistically significant except for 4-methoxyglucobrassicin due to large variation among repetitions (**Figure 3A**). The fresh watercress samples derived from distinct production areas considerably differed in glucosinolate content. Freshness and toughness of the leaf tissues of the samples should also affect the timing when brining NaCl reaches the tissue cells and the degradation rate of glucosinolates. Verkerk et al. reported that chopping and storage of broccoli and cabbage resulted in an increase of indole glucosinolates (*23*). An increase of all glucosinolates analyzed was observed in one of three samples after 1−2 days of pickling, suggesting that such a response occurs under salt stress or compression during the pickling process, whereas they, except 4-hydroxyglucobrassicin, decreased in other samples. These factors made detection of difference by the statistical analysis difficult. Among the glucosinolates analyzed, only 4-methoxyglucobrassicin decreased significantly with increasing pickling period. This glucosinolate was stably detected in all samples, resulting in the statistical significance. After 7 days, the ratio of indole glucosinolates to total glucosinolates was 78%, whereas it was 17% before pickling, suggesting that indole glucosinolates were abundant in the pickled watercress.

In experiment 2, all glucosinolates except 4-hydroxyglucobrassicin significantly ($p < 0.05$) decreased after 7 days of pickling (**Figure 3B**). Blanching of watercress slows or stops enzyme reactions involved in the synthesis and degradation of

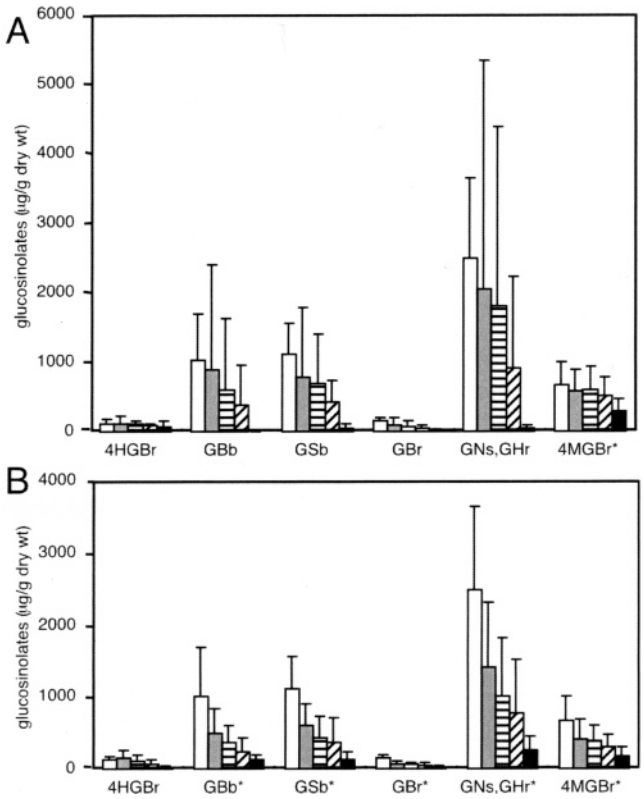

Figure 3. Effects of pickling process of watercress on glucosinolate content: (**A**) fresh watercress and (**B**) blanched watercress were soaked in 3% NaCl, and the glucosinolate content was examined (see **Table 1** for abbreviations). The glucosinolate contents on days 0, 1, 2, 3, and 7 are represented as white, gray, horizontal striped, hatched, and black bars, respectively. Values are expressed as mean ± standard errors ($n = 3$). Each glucosinolate with an asterisk significantly decreased during 7 days of pickling ($p < 0.05$).

glucosinolates. At the same time it disrupts the membrane structure and facilitates reaction of the residual myrosinase with glucosinolates. These effects resulted in reduction of variation factors between samples, and thus a decrease of the glucosinolates by pickling became significant. The brine was also analyzed by HPLC, but no glucosinolates were detected, suggesting that most glucosinolates were retained in the tissues during the pickling process. In the experiment 2, the ratio of indole glucosinolates to total glucosinolates increased to 181% of the initial level after 7 days of pickling. There was no statistically significant difference between mean values of total glucosinolates in experiments 1 and 2, whereas they were 468 and 747 μg/g of dry weight, respectively. The effect of blanching might reduce degradation of glucosinolates.

Glucosinolates in Nozawana-zuke and Their Myrosinase Susceptibility. In the above simulated pickling experiments, increase of the relative ratio of indole glucosinolates to total glucosinolates was observed after 7 days of pickling. We thus compared the glucosinolate content of nozawana-zuke (pickled nozawana) and fresh nozawana from a farm. As shown in **Table 2**, glucobrassicin and gluconasturtiin were the major glucosinolates in nozawana-zuke. Glucoberteroin and 4-methoxyglucobrassicin were also detected, whereas other glucosinolates abundant in the fresh nozawana, such as glucoerucin, were not detected. As described above, chopping and storage of broccoli and cabbage resulted in an increase of indole glucosinolates (*23*). If an increase of glucosinolates occurs during the pickling process, it can counteract the myrosinase-mediated hydrolysis. To examine whether such an increase of glucosinolates by salt stress is a cause of the apparent stability of some glucosinolates or whether susceptibility of the glucosinolates to myrosinase determines their stability in the pickling process, each glucosinolate purified from nozawana was treated with myrosinase in the presence of ascorbic acid. As shown in **Table 3**, 4-methoxyglucobrassicin was revealed to be highly resistant to myrosinase. It is consistent with the observation that 4-methoxyglucobrassicin was stably detected in all samples of simulation experiments. There are three groups of myrosinase in plant tissues. The myrosinase used in this experiment belongs to the MA family and presents as a soluble dimer (140 kDa), whereas the MB and MC families of myrosinases are found in complexes with several other proteins (*24*). As an obvious decrease of 4-methoxyglucobrassicin was observed during the simulation of pickling, the MB and/or MC family may degrade this substrate. There also may be other enzymes that can degrade the glucosinolates. On the other hand, glucobrassicin was susceptible to the myrosinase. Survival of this glucosinolate in nozawana-zuke suggests that glucobrassicin might have formed during the pickling process.

Table 2. Glucosinolate Contents in Fresh Leaves and Pickled Products of Nozawana

	glucosinolate (μg/g of dry wt)	
common name	nozawana (fresh leaves)	nozawana-zuke
gluconapin	763 ± 61	11 ± 19
4-hydroxyglucobrassicin	131 ± 23	5.8 ± 5.2
glucoerucin	2519 ± 417	0.0 ± 0.0
glucobrassicin	474 ± 98	199 ± 173
gluconasturtiin	568 ± 331	167 ± 160
glucoberteroin	1026 ± 295	57 ± 50
4-methoxyglucobrassicin	768 ± 79	35 ± 33

Table 3. Effect of Myrosinase Digestion on Each Glucosinolate in Nozawana

common name	remaining rate (%) after myrosinase digestion	conversion rate to degradation produdct (%)
gluconapin	0 ± 0	
4-hydroxyglucobrassicin	0 ± 0	51 ± 0[a]
glucoerucin	0 ± 0	
glucobrassicin	10 ± 2.6	30 ± 1.8
gluconasturtiin	16 ± 0	
glucoberteroin	31 ± 28	
4-methoxyglucobrassicin	86 ± 6.9	

[a] Identified as hydroxyascorbigen.

In addition to the glucosinolate peak, an extra peak was observed in the HPLC analysis of myrosinase-treated 4-hydroxyglucobrassicin or myrosinase-treated glucobrassicin. On LC-MS analysis, this degradation product of 4-hydroxyglucobrassicin had a molecular weight of 305 and was estimated as hydroxyascorbigen. Consistent with the previous observation (*25*), no other peaks of intermediates were observed, suggesting that hydroxyascorbigen was formed directly from ascorbate and an unstable hydrolyzed product of 4-hydroxyglucobrassicin (**Figure 1**). The degradation product of glucobrassicin, ascorbigen, was not detected by LC-MS analysis.

Indole glucosinolates give rise to a variety of products on hydrolysis by myrosinase (**Figure 1**). During ingestion, indole glucosinolates are converted in the body of the host to various products, including indole-3-carbinol and ascorbigens (*26*, *27*). Data from in vitro and animal experiments suggest that natural indole derivatives generated from cruciferous vegetables possess cancer chemopreventive properties (*28*, *29*). Indole glucosino-

lates are also thought to be involved in the modulation of xenobiotic metabolism. Intact indole glucosinolates were more powerful inducers of specific cytochrome P-450 and glutathione *S*-transferase isoforms than the in vitro myrosinase-degraded indole glucosinolates (*30*). Certain indole glucosinolates form oligomeric compounds, such as 3,3′-diindolylmethane, under acidic conditions in vitro or in the stomach. This compound has shown a protective effect against tumor induction by some carcinogens but has also been observed to promote tumor development in animal models (*31*). Indole glucosinolates also form nitroso compounds by treatment with nitrite, which are thought to have negative health effects (*32*, *33*). Traditionally, the pickling process was used to preserve vegetables, resulting in alleviation of the pungent flavor of cruciferous vegetables. We should note that a major health problem in Japan is high blood pressure and stroke along with cancer of the stomach, from excessive intake of salt and salted, pickled foods (*34*). Thus, the benefits and risks of pickled vegetables containing glucosinolates and their degradation products on human health should be investigated further, together with improvement of the manufacturing process.

Two vegetables used in this study, watercress and nozawana, are minor vegetables having limited usage; however, they contain many kinds and high concentrations of glucosinolates. From this point of view, there must be additional cruciferous vegetables containing beneficial compounds that are cultivated and processed locally in Japan and other countries. Although the yield and the distribution system of such local products may be a problem for mass consumption, they can sometimes be promoted in the local area. By reconsidering conventional ways of cooking or processing and developing new ways of cooking or processing to retain glucosinolates, we may be able to increase glucosinolate intake.

In this study it was shown that, in the case of pickled products, a shorter maturation period retains more glucosinolates. The ratio of indole glucosinolates increased during the pickling period due to a stress response and/or their myrosinase resistance, whereas total glucosinolates decreased. We also showed the possibility that blanching stops the decrease of glucosinolate content during the pickling process. By developing new pickling processes that repress myrosinase activity, pickled cruciferous vegetables would be good sources of glucosinolates and contribute to a balanced diet.

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