JARQ 42 (2), 109−114 (2008) http://www.jircas.affrc.go.jp

A Chromatographic Method for Separating and Identifying Intact 4-Methylthio-3-Butenyl Glucosinolate in Japanese Radish (*Raphanus sativus* L.)

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Abstract

To isolate and measure intact 4-methylthio-3-butenyl glucosinolate (4MTB-GSL) in Japanese radish, we applied a paired ion chromatography method that uses tetradecylammonium ions (TDA) as counter ions. 4MTB-GSL was isolated as ammonium salts from the root of a Japanese radish and identified on the basis of myrosinase treatment, aglycon detection, negative fast atom bombardment (FAB)-mass spectra, and a UV spectrum. The linearity of the prepared standard solution and recovery of that added to Japanese radish extracts were high.

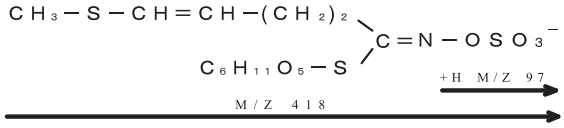
Discipline: Agricultural chemicals
Additional key words: FAB-MS, kaiware daikon, paired ion, sprout, UV spectrum

Introduction

When raw crucifers are grated, chewed, or chopped, glucosinolates (GSLs) come into contact with endogenous myrosinase (thioglucoside glucohydrolase EC 3.2. 3.1), and breakdown products, including isothiocyanates (ITCs), nitriles, and thiocyanate, are released (e.g., see Itoh[13]). Japan produces a large crop of Japanese radish (*Raphanus sativus* L), a crucifer. Intact roots of 109 radish cultivars that were analyzed for GLSs were found to contain mainly *trans*-4-methylthio-3-butenyl glucosinolate (4MTB-GSL) (Fig. 1(a)) and Japanese radishes showed high concentrations (241 μmole/100 g fresh weight)[2,24]. They only contain very small amounts of other GSLs[1,2]. The main GSL breakdown product is 4-methylthio-3-butenyl isothiocyanate (4MTB-ITC)[12-15,18], a pungent compound[7]. In a study of the antimutagenic activity of Japanese radish using a UV-induced mutation assay with *Escherichia coli*, a correlation was found between anti-mutagenicity and the amount of 4MTB-ITC used[17]. 4MTB-ITC content can be influenced by pre-harvest conditions[14,15,18]. Thus, GSLs that break down into ITCs are interesting as mutagen inhibitors.

Many methods of analyzing GSLs have been developed. Desulfo-GSLs are analyzed using high-performance liquid chromatography (HPLC)[11,16], trimethylsilyl derivatives are analyzed using gas chromatography (GC)[19,23], and aglycon is analyzed using GC or spectrophotometry[2,3]. Thus, existing methods need chemical modification by enzymatic treatment and/or silylation and they are time consuming. Simple and direct methods for analyzing GSLs content are available. The methods utilize paired ion chromatography that use tetraalkylammo-

(a) 4-Methylthio-3-butenyl glucosinolate



(b) Sinigrin.

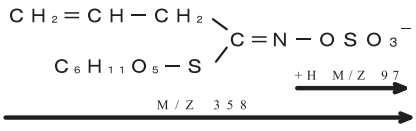


Fig. 1. Structure of glucosinolates

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Received 12 October 2006; accepted 8 Novemer 2007.

nium ions as counter ions[9,20]. Moreover, replacing the counter ions, tetradecyl ammonium ions (TDA) by ammonium ions permits direct bioassay, mass spectrometry (MS) and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy[20]. However, the direct analytical method for 4MTB-GSL in Japanese radish has not been reported. Trifluoroacetate as paired ion reagent would degrade GSLs rapidly under the low pH character because GSLs are hydrolysed in strong acid solution[4].

MS can confirm already-known GSLs with less labor than NMR because MS is more sensitive than NMR. Although the GSLs and desulfo-GSLs of *Brassicaceae* have been analyzed with electron impact (EI) induced mass spectra, their mass spectra contained no information on the molecular ions[6]. On the other hand, negative fast atom bombardment (FAB)-MS is available to detect the molecular ion, $[M-H]^-$ of GSL[5,20]. However, negative FAB-mass spectrum of 4MTB-GSL has not been reported.

It is difficult to get 4MTB-GSL because the reagent has not been sold. Though the isolation procedure as the potassium salts was reported, it is complicated and time consuming[24].

As mentioned above, not having an intact, convenient analytical method and isolation procedure of 4MTB-GSL impede the research of 4MTB-GSL in Japanese radish. Therefore, the objective of this study was to develop the paired ion method that uses TDA as counter ions for analyzing intact 4MTB-GSL in Japanese radish, measuring the negative FAB- mass spectrum and describing the isolation procedure as the ammonium salts.

Materials and methods

4MTB-GSL was extracted from the root of a Japanese radish (cv.' Karamaru ', Sakata Seed, Japan) by addition of 500 ml of methanol (Wako Pure Chemical Industries, special grade, Japan) to 368 g of the peeled and diced root. This mixture was homogenized, and the methanol extract was filtered before the fractionating using HPLC. HPLC consisted of a pump, an injector, a column oven (Japan Spectroscopic Co., Japan), and a photo diode array detector (Toso, PD-8020, Japan). The conditions used to fractionate the filtered extract were as follows. The eluent was 65% acetonitrile (Wako Pure Chemical Industries, HPLC grade, Japan) and 35% water (Millipore, Elix-UV-3, Japan) (V/V) containing 2.5 mM tetradecylammonium bromide (TDAB) (Tokyo Kasei Kogyo, Japan) (pH 6.0), used at a flow rate of 3.0 mL/min with a column at room temperature. The column was a Capcellpak C8 UG120, particle size 5μm, i.d. 10 mm, and length 250 mm (Shiseido, Japan). Detection occurred at 225 nm. The fractionated 4MTB-GSL was dried using an evaporator (Sibata, RE111, Japan) and purified as ammonium salts. One-half to 1 mL of chloroform (Wako Pure Chemical Industries, special grade, Japan) was added to the dried material followed by 250 μL of 3 mM ammonium sulfate (Wako Pure Chemical Industries, special grade, Japan) adjusted with NH_4OH (Wako Pure Chemical Industries, special grade, Japan) to pH 7.0. After mixing, the aqueous phase was separated and reextracted twice with chloroform. The aqueous phase was removed and dried using the evaporator. The residue was suspended in 300 μL of methanol, and the insoluble ammonium sulfate was removed by centrifugation. The methanol phase, containing 4MTB-GSL was dried using the evaporator[20]. The flow sheet of 4MTB-GSL isolation is shown in Fig. 2.

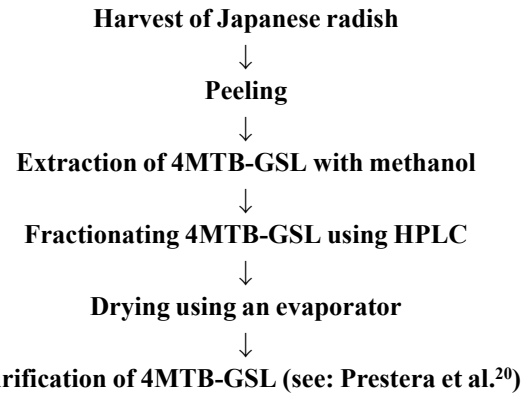

Fig. 2. Flow sheet of 4MTB-GSL isolation

To detect the aglycon, 4MTB-ITC, in an aqueous solution of the prepared 4MTB-GSL as ammonium salts treated with exogenous myrosinase (Sigma, thioglucosidase from *Sinapis alba* seed, USA), the solution was extracted with diethylether (Wako Pure Chemical Industries, diethyl ether 2000, pesticide analysis grade, Japan). The extract was injected into a gas chromatograph (GC; Shimadzu 15A, Japan) and the retention time of the peak was compared to the standard[12-15,18]. The GC was equipped with a capillary column (J & W Scientific, DB5, length 30 m, i.d. 0.25 mm, film thickness 0.25 μm) and a flame photometric detector (FPD). The injector and detector were set at 200°C and the column oven increased from 70 to 200°C (2°C/min) followed by 20 min at 200°C. To determine if the prepared 4MTB-GSL were contaminated with 3-indolylmethyl GSL, the breakdown product, thiocyanate, in the solution was also analyzed using HPLC[10].

To confirm the molecular weight of the prepared 4MTB-GSL, the $[M-H]^-$ (m/z 418) was analyzed using

negative FAB-MS (JEOL, The MStation JMS-700, Japan). Samples in methanol (1 μL) were prepared by adding each to 1 μL of a glycerol (Tokyo Kasei Kogyo, FAB-MS grade, Japan)-based matrix previously placed on a probe tip. The accelerating voltage of the source was 10 kV. The collision gas was xenon (6 keV). Then mass spectrum of sinigrin (Indofine Chemical Company, USA), another glucosinolate was also measured to compare with that of 4MTB-GSL.

Next, we quantified intact 4MTB-GSL in Japanese radish using HPLC. To extract 4MTB-GSL, 40 mL of boiled methanol were added to 5 g fresh weight of cotyledon and hypocotyls (kaiware daikon) purchased at a retail store or 11 g root that was stored for about 3 months and incubated for 5 min at 65°C. The mixtures were then homogenized, and the methanol extract was filtered to a final volume of 50 mL by washing the particles with methanol. The analytical conditions were as follows. The eluent was 65% acetonitrile and 35% water (V/V) containing 2.5 mM TDAB at a flow rate of 1.5 mL/min with a column temperature of 35°C. The column was a Capcellpak C8 UG120, particle size 5 μm, i.d. 4.6 mm, and length 250 mm (Shiseido, Japan). Kaiware daikon extract was analyzed using a manual injector, while the root extract was analyzed using an autosampler. Detection occurred at 225 nm using an ultraviolet-visible detector for quantification (Japan Spectroscopic Co., UV-970, Japan) or at 225-260 nm using the photodiode array detector for measuring the spectrum. The prepared 4MTB-GSL as ammonium salts was solved in water at 100 ppm (standard solution). The aliquot volume was injected into HPLC and the linearity was checked. Considering the results, when the standard solution was added to the two extracts, the recovery was examined respectively.

Results and discussion

When the standard solution was treated with exogenous myrosinase, the peak almost disappeared on the HPLC chromatogram (Fig. 3). The other prominent peaks came from the blank (water) or resulted from myrosinase treatment. The lost peak appeared to contain GSL. Only the one peak for 4MTB-ITC was detected on the GC chromatogram of the diethylether extract (Fig. 4). It is supposed that this peak consists of almost only *trans*- form[2,12-15,18,24]. Thus, aglycon from 4MTB-GSL was detected. Next, the molecular weight of 4MTB-GSL was analyzed. The intensity of $[M-H]^-$ (m/z 418) was increased by acidifying the glycerol matrix with hydrochloric acid as reported by Fenwick et al.[5]. The other peaks of m/z at 97 and 189 and some intensive peaks (m/z 91, 183, 275) were associated with the sulfate moiety $[HSO_4^-]$ of the GSL

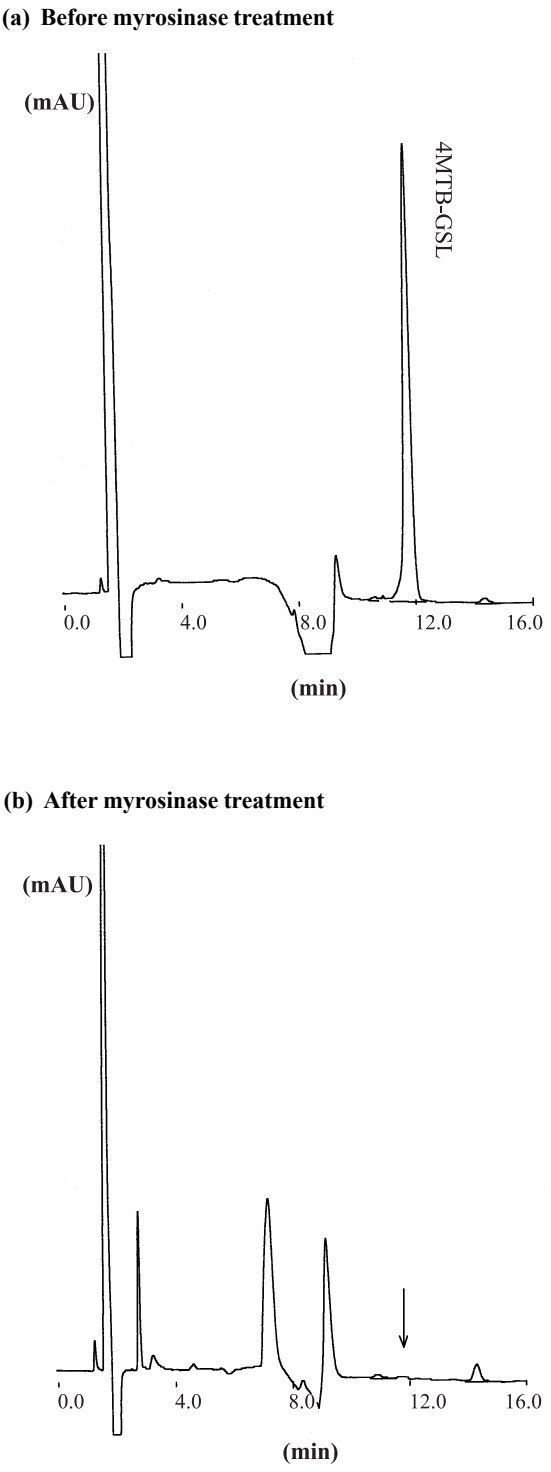

Fig. 3. HPLC chromatogram of the standard solution of 4MTB-GSL・NH_4
The conditions of HPLC analysis were as follows. Eluent: acetonitrile 65% and water 35% (V/V) containing 2.5 mM TDAB; Flow rate: 1.5 mL / min; Column temperature: 35°C; Column: Shiseido Capcellpak C8 UG120, particle size 5 μm, i.d. 4.6 mm, length 250 mm. Detection: 225 nm. An arrow in (b) shows the disappeared 4MTB-GSL when treated with exogenous myrosinase.

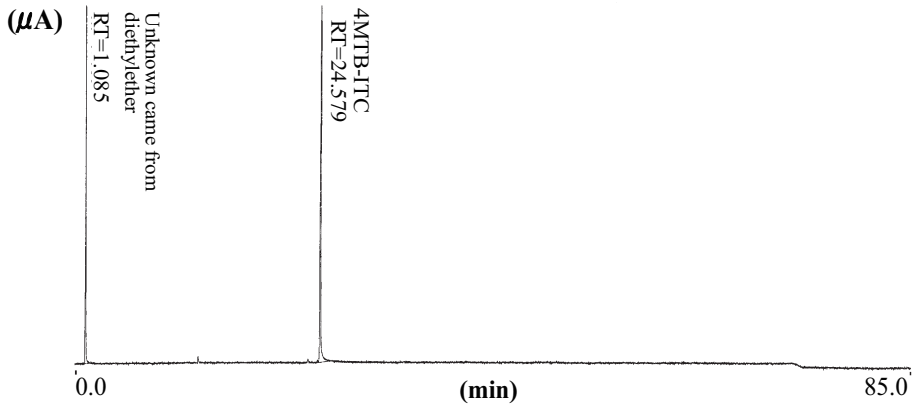

Fig. 4. GC chromatogram of 4MTB-ITC
4MTB-ITC detection from the standard solution of 4MTB-GSL・NH_4 treated with exogenous myrosinase. The retention time was consistent with the standard, 4MTB-ITC using GC-FPD. GC was equipped with a capillary column (J & W Scientific, DB5, length 30 m, i.d. 0.25 mm, film thickness 0.25 μm). The injector and detector were set at 200°C, column oven from 70 to 200°C (2°C/min) followed by 20 min at 200°C.

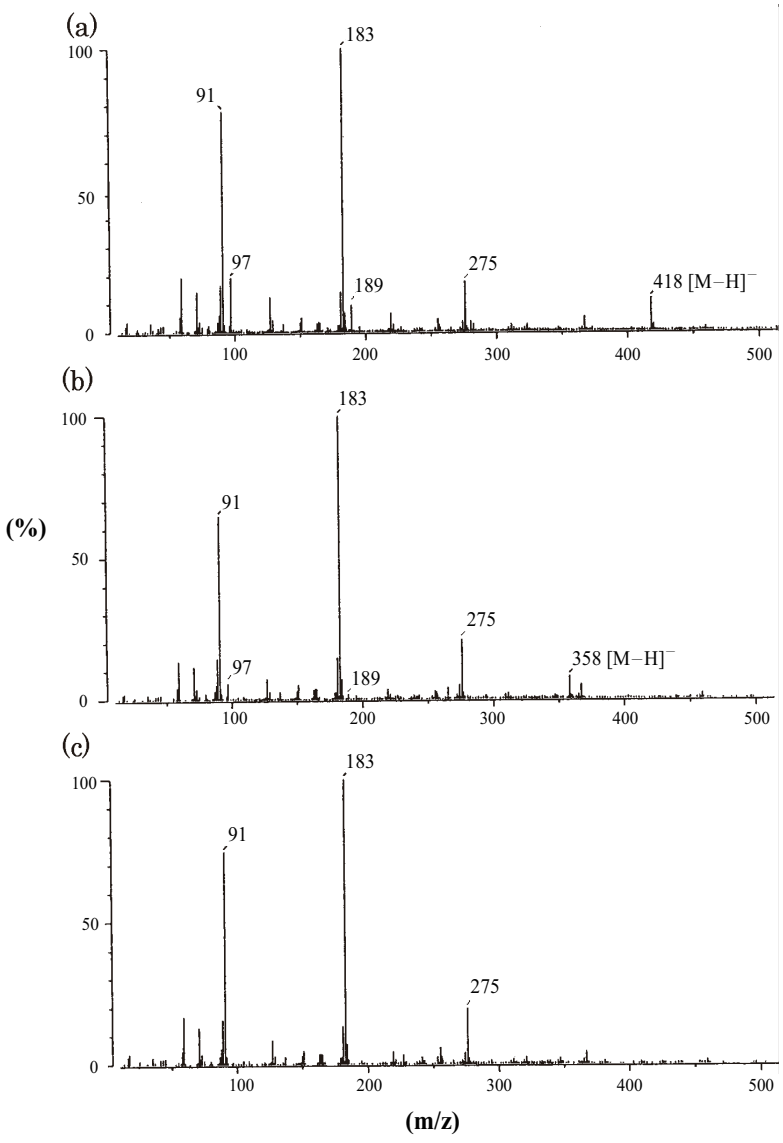

Fig. 5. Negative FAB-mass spectra
(a) 4MTB-GSL, (b) sinigrin, (c) acidified glycerol matrix.

and the GSL and glycerol matrix, respectively, as well as with sinigrin (Figs. 1, 5) as shown by Sakushima et al.[21]. The UV spectrum of 4MTB-GSL was similar to that of desulfo 4MTB-GSL[22,24] (Fig. 6). Minor amounts of 3-indolymethyl and 4-methylsulfinyl-3-butenyl GSL may be detected simultaneously. However, the thiocyanate ion, which demonstrates contamination by 3-indolylmethyl GSL[2,8], was not detected in the standard solution treated with exogenous myrosinase, and if 4-methylsulfinyl-3-butenyl GSL were also in the aqueous solution, the aglycon would be detected by the GC-FPD that is sensitive to sulfur and has high resolution. Thus, the lost peak on HPLC appeared to contain only 4MTB-GSL. The yield of 4MTB-GSL ammonium salts was 0.003%. However, two small peaks at 10.9 min and 14.4 min were not 4MTB-GSL (Fig. 3(a)) and appeared to be contamination from fractionation of 4MTB-GSL. Therefore, the purity was calculated at 98.0% by the peak area. The linearity of 4MTB-GSL was 0.999 between 0.14 and 1.37 µg (Fig. 7). The contents of 4MTB-GSL were 816.1 ± 22.4 and 177.6 ± 0.9 µmole/100 g fresh weight for the kaiware daikon and for the root, respectively. The recovery rates were 102.5 ± 5.5 and 102.0 ± 0.3% (n = 3) for the kaiware daikon and for the root, respectively. TDA was used as counter ions when analyzing other GSLs[20].

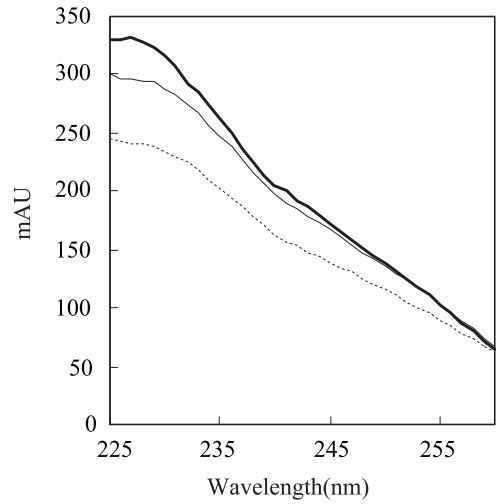

Fig. 6. UV spectra of 4MTB-GSL
The UV spectrum of 4MTB-GSL was similar to that of desulfo 4MTB-GSL[22,24].
·····: Root, ———: Kaiware daikon,
———: 4MTB-GSL.

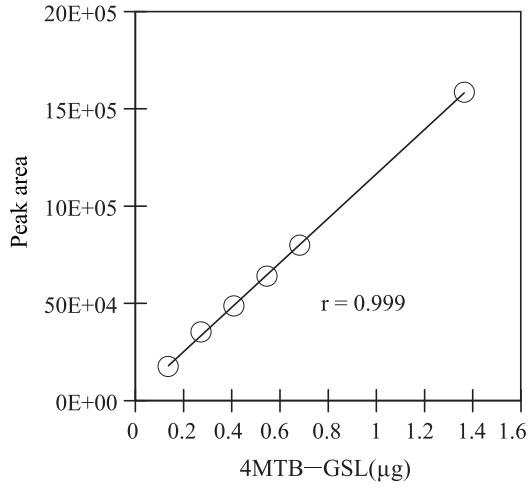

Fig. 7. The linearity of 4MTB-GSL
A correlation coefficient between 0.14 and 1.37 µg was 0.999.

As mentioned above, we applied a paired ion chromatographic method that uses TDA as counter ions to isolate and analyze intact 4MTB-GSL in Japanese radish.

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