Nomal-phase High-performance Liquid Chromatography in the Study of 4-Methylthio-3-butenyl Isothiocyanate from Daikon (Raphanus sativus)

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Normal-phase High-performance Liquid Chromatography in the Study of 4-Methylthio-3-butenyl Isothiocyanate from Daikon (*Raphanus sativus*)

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I Introduction

Daikon (Japanese white radish, *Raphanus sativus* L.) is eaten throughout Japan. Japanese people often eat daikon *oroshi* (grated raw daikon) with soy sauce, *nametake* (Japanese mushroom), *tempura* (a traditional Japanese dish of fried fish and vegetables), or grilled fish. 4-Methylthio-3-butenyl isothiocyanate (Fig. 1) is the principal isothiocyanate in grated daikon. When daikon is grated, this substance is produced from 4-methylthio-3-butenyl glucosinolate by the action of myrosinase (*β*-thioglucosidase) (Fahey et al., 2001). It gives daikon *oroshi* its pungent odor and biting taste (Nakamura et al., 2001). The characteristic yellow of *takuan* (salted-fermented daikon, a traditional Japanese daikon product) comes from a major degradation product of 4-methylthio-3-butenyl isothiocyanate (Ozawa et al., 1990, Ozawa et al., 1999). Thus 4-methylthio-3-butenyl isothiocyanate is an important odor, taste, and color factor in the cooking and processing of daikon.

Many investigations have found that some phytochemicals have chemopreventive activity against cancer. Isothiocyanates are thought to be an important class of phytochemicals with this capacity (Nakamura et al., 2006). 4-Methylthio-3-butenyl isothiocyanate induces detoxification enzymes in the HepG2 human hepatoma cell line (Hanlon et al., 2007). It also reduces cell proliferation in a dose-dependent manner and induces apoptosis in three human colon carcinoma cell lines (Papi et al., 2008). The chemopreventive effect of 4-methylthio-3-butenyl isothiocyanate has thus recently attracted considerable attention.

For these reasons, an accurate analysis of 4-methylthio-3-butenyl isothiocyanate is very important for evaluating the quality and chemopreventive effects of daikon. The prevailing method used to quantify 4-methylthio-3-butenyl isothiocyanate is gas chromatography (GC) (Nakamura et al., 2001, Okano et al., 1990). However, not all laboratories are equipped with GC apparatus. For our research, we needed an easy procedure using high-performance liquid chromatography (HPLC) to quantify 4-methylthio-3-butenyl isothiocyanate in a

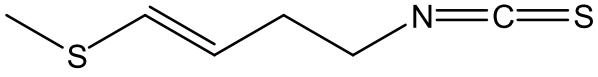

Fig. 1 Structure of 4-methylthio-3-butenyl isothiocyanate.

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daikon extract. Unfortunately, reverse-phase HPLC with an aqueous mobile phase cannot be used to precisely quantify 4-methylthio-3-butenyl isothiocyanate, because this compound is spontaneously converted into raphanusanins in aqueous solution (Kosemura et al., 1993). To solve this problem, we developed a normal-phase HPLC that used silica and an n-hexane/2-propanol isocratic mobile phase. This analysis does not need an aqueous solution and enables powerful quantitation that is complementary to GC analysis of 4-methylthio-3-butenyl isothiocyanate.

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Ⅱ Materials and Methods

1 Materials

Daikon was purchased from a local supermarket. The organic solvents used in HPLC were HPLC grade.

2 Preparation of 4-methylthio-3-butenyl isothiocyanate standard

4-Methylthio-3-butenyl isothiocyanate from daikon was purified in our laboratory. Fresh daikon roots (5.8 kg) were grated in a food processor at room temperature and then centrifuged at 10,000 × g for 3 min at 25 ˚C. The supernatant (4.4 l) was shaken with n-hexane (4.4 l). The n-hexane extract (528 mg) was applied to a preparative HPLC system (Develosil 30-3 silica column, 250 mm × 20 mm; Nomura Chemical, Aichi, Japan) at 40 ˚C. The column was eluted at a flow rate of 10.0 ml/min with an isocratic mobile phase (n-hexane/ethyl acetate, 92:8). The eluate was monitored by ultraviolet (UV) detection at a wavelength of 230 nm. The isolated 4-methylthio-3-butenyl isothiocyanate (341 mg) was checked by nuclear magnetic resonance (NMR) and mass spectrometry analyses (Kosemura et al., 1993). The ^{1}H and ^{13}C NMR spectra of the isolated 4-methylthio-3-butenyl isothiocyanate in chloroform-d (solvent as an internal standard, δ 7.26 and 77.0, respectively) were recorded on an EX270 spectrometer (JEOL, Tokyo, Japan) at 270 and 67.5 MHz, respectively. The electron impact ionization-mass spectrum of the isolated 4-methylthio-3-butenyl isothiocyanate was obtained with a JMS-700 mass spectrometer (JEOL).

3 Measurement of UV spectrum of 4-methylthio-3-butenyl isothiocyanate

The UV spectrum of 4-methylthio-3-butenyl isothiocyanate dissolved in n-hexane/2-propanol (99.5:0.5) was measured with a U-2810 spectrophotometer (Hitachi, Tokyo, Japan).

4 n-Hexane extraction of 4-methylthio-3-butenyl isothiocyanate from daikon

The method developed by Coogan et al. (2001) for extracting 4-methylthio-3-butenyl isothiocyanate from daikon was modified for this study. The daikon sample was grated in a ForceMill (Osaka Chemical, Osaka, Japan) to convert 4-methylthio-3-butenyl glucosinolate to 4-methylthio-3-butenyl isothiocyanate. The pulp of the grated sample was centrifuged at 12,000 × g for 5 min at 25 ˚C. Daikon juice was obtained from the supernatant by filtration. Juice (6 ml) and n-hexane (2 m1) were placed into a stoppered conical tube and then shaken vigorously for 30 s on a vortex mixer. The tube was centrifuged at 1400 × g for 5 min at 25 ˚C, and the n-hexane fraction was then collected. The aqueous phase was extracted with n-hexane (2 m1) twice as above. The pooled n-hexane fraction was removed into an HPLC vial for HPLC-UV analysis.

5 HPLC-UV analysis of 4-methylthio-3-butenyl isothiocyanate

The HPLC instrument was an LC-2000Plus Series System (JASCO, Tokyo, Japan). We used a Develosil 30-3, silica, 250 mm × 4.6 mm column (Nomura Chemical). The mobile phase was isocratic (n-hexane/2-propanol, 99.5:0.5). The column was operated at 40 ˚C with a flow rate of 1.0 ml/min. The wavelength for detecting 4-methylthio-3-butenyl isothiocyanate was 232 nm and the data were processed with ChromNAV software (JASCO). Quantitation was based on a 4-methylthio-3-butenyl isothiocyanate standard.

Ⅲ Results and Discussion

A standard of 4-methylthio-3-butenyl isothiocyanate was purified from daikon as described in the Materials and Methods section. Because 4-methylthio-3-butenyl isothiocyanate spontaneously changes into other compounds in aqueous solution, n-hexane extraction from the supernatant of grated daikon is an important step to avoid the loss of 4-methylthio-3-butenyl isothiocyanate, which is stable in n-hexane (Kosemura et al., 1993). Preparative normal-phase HPLC facilitated isolation of 4-methylthio-3-butenyl isothiocyanate from the n-hexane extract. Starting with 5.8 kg of fresh daikon roots, we obtained 341 mg of pure 4-methylthio-3-butenyl isothiocyanate (*trans* form), as assessed by NMR (Fig. 2) and mass spectroscopies (Kosemura et al., 1993), by using only two purification steps. This purification procedure thus enabled the large-scale isolation of

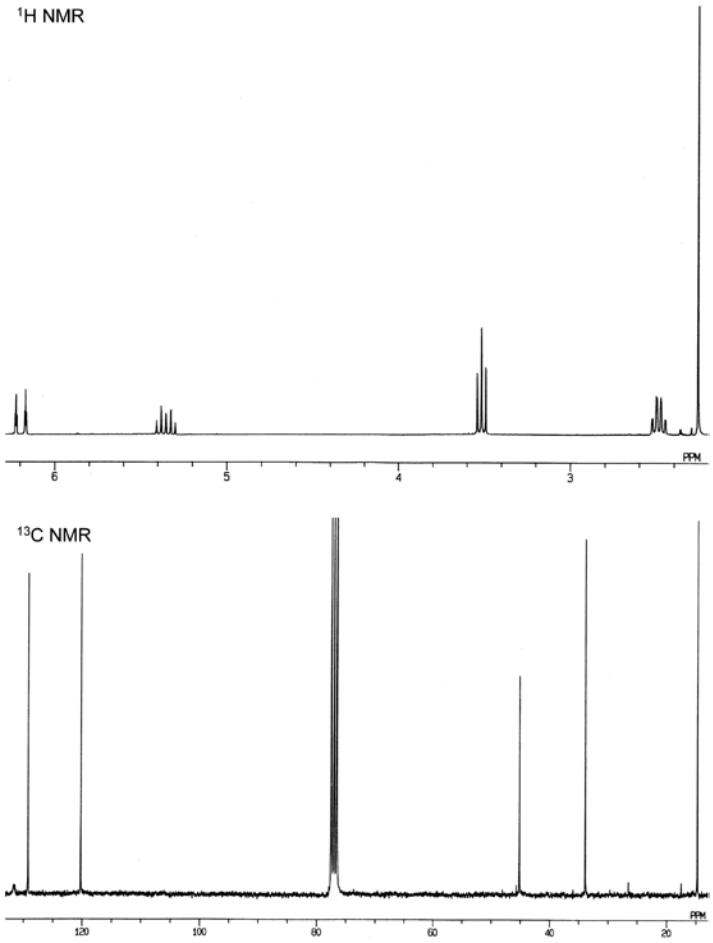

Fig. 2 ^{1}H and ^{13}C NMR spectra of 4-methylthio-3-butenyl isothiocyanate isolated from daikon.

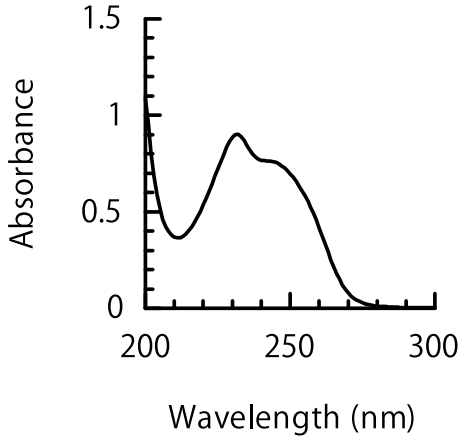

Fig. 3 UV spectrum of 4-methylthio-3-butenyl isothiocyanate in n-hexane/2-propanol (99.5:0.5).

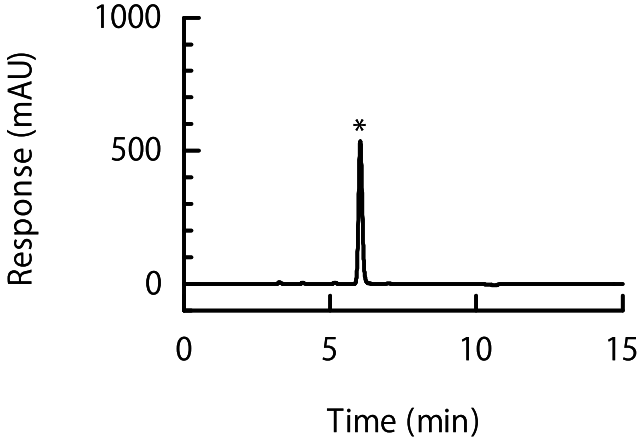

Fig. 4 HPLC-UV chromatogram of 4-methylthio-3-butenyl isothiocyanate extracted with n-hexane from daikon. Asterisk shows the peak of this compound.

4-methylthio-3-butenyl isothiocyanate to be used as a standard.

We examined the UV spectrum of 4-methylthio-3-butenyl isothiocyanate dissolved in an HPLC mobile phase (n-hexane/2-propanol, 99.5:0.5) (Fig. 3). The maximal absorption wavelength was 232 nm; this was therefore chosen as the optimum wavelength for detecting 4-methylthio-3-butenyl isothiocyanate in HPLC analysis. 4-Methylthio-3-butenyl isothiocyanate was easily recognized as the tallest peak in the HPLC chromatogram of the n-hexane extract (Fig. 4). The 4-methylthio-3-butenyl isothiocyanate peak was well separated from the matrix signals in the daikon extract. The coefficients of variation of the area counts and the retention time for ten 80 µl injections of a 51 nmol 4-methylthio-3-butenyl isothiocyanate standard were 1.9% and 1.7%, respectively, thus providing evidence of the robustness of this chromatographic system. The detection limit of 4-methylthio-3-butenyl isothiocyanate by this HPLC analysis was under 10 pmol.

For the analysis of 4-methylthio-3-butenyl isothiocyanate, we developed normal-phase HPLC that used silica and an n-hexane/2-propanol isocratic mobile phase. For analyzing 4-methylthio-3-butenyl isothiocyanate in daikon *oroshi*, the n-hexane extraction step is very important to stop the activity of myrosinase and avoid the loss of the target compound. Unlike with reverse-phase HPLC, the n-hexane extract solution can be directly injected into this normal-phase HPLC system without solvent substitution. Moreover, this method solves the problem of 4-methylthio-3-butenyl isothiocyanate being unstable in aqueous solution. We believe that this method will prove valuable to other researchers studying daikon.

Summary

4-Methylthio-3-butenyl isothiocyanate is an important odor, taste, and color factor in the cooking and processing of daikon (Japanese white radish, *Raphanus sativus* L.) and has chemopreventive activity against cancer. Because it is spontaneously converted into other compounds in aqueous solution, it cannot be properly analyzed by reverse-phase high-performance liquid chromatography (HPLC) with an aqueous mobile phase. The normal-phase HPLC that we developed with silica and an n-hexane/2-propanol (99.5:0.5) isocratic mobile phase solves this problem. It is a powerful quantitative method that is complementary to gas chromatography analysis of 4-methylthio-3-butenyl isothiocyanate.

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ダイコン （*Raphanus sativus*） の 4- メチルチオ -3- ブテニルイソチオシアネート研究のための 順相高速液体クロマトグラフィーの適用

一法師 克成* ・ 石田 正彦** ・ 竹内 敦子 ・ 東 敬子

摘 要

4- メチルチオ -3- ブテニルイソチオシアネートは， ダイコン （*Raphanus sativus* L.） の調理および加工時の重要な匂い， 味および色の因子であり， 化学予防作用を示すことが報告されている．4- メチルチオ -3- ブテニルイソチオシアネートは水溶液中で自然に他の物質へ変化するので， 移動相に水を用いる逆相高速液体クロマトグラフィー （HPLC） では正確な分析ができない． 開発したシリカゲルカラムと n- ヘキサン /2- プロパノール （99.5:0.5， アイソクラティック） の移動相を用いる順相 HPLC は， この問題を解決し， ガスクロマトグラフィー分析を補完する 4- メチルチオ -3- ブテニルイソチオシアネートの強力な定量法を提供する．

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