Analysis of Lactucopicrins (Bitter Compounds) in Lettuce by High-Performance Liquid Chromatography

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I Introduction

Because of its flavor and texture, lettuce (*Lactuca sativa*) is one of the most popular salad vegetables in Japan, even though no unique nutritive values have been discovered in lettuce, and its vitamin C and β-carotene contents are much lower than those of spinach and other leafy vegetables.

The Committee for Investigation of the Palatability of Vegetables (Yasai no Oishisa Kento Iinkai) has been collecting information on the quality of Japanese vegetables; for example, the committee conducted organoleptic tests on several varieties of crisp-head lettuce in 2007 (Horie, 2008). Although higher sugar content scores favorably for most vegetables, no relationship between the sensory evaluated taste scores and sugar content was observed for crisp-head lettuce in these tests. Bitter lettuce samples were evaluated unfavorably (Horie, 2008). For the physicochemical evaluation of lettuce quality, analysis of the amounts of bitter compounds is important.

Lettuce contains sesquiterpene lactones (SLs), which are reported to be related to bitter taste (Price et al. 1990). Sessa et al. (2000) reported that the most abundant SL in lettuce is lactucopicrin 15-oxalate (LO) and that small amounts of lactucopicrin are also present. Recently, Arakawa et al. (2008) compared the SL contents of various *Lactuca* species cultivated in Japan and reported that the major SL is lactucopicrin; these researchers did not mention the LO content. Therefore, clarification of whether lettuce leaves cultivated in Japan contain LO is necessary.

Arakawa et al. (2007) modified the sample preparation methods for the analysis of SLs that had been reported by Price et al. (1990), shortening the preparation time from 13 h to 2 h, but the necessary preparation is still tedious. A simple method for rapid analysis is required.

Therefore, the aims of this research were (1) to establish a simple method for the analysis of SLs in lettuce, (2) to confirm the presence of LO in Japanese lettuce, and (3) to investigate the relationship between SL content and bitterness.

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II Materials and Methods

1 Reagents

The SLs present in lettuce are not commercially available, so they must be purified before analytical conditions can be developed. Because the stems of bolted crisp-head lettuce are very bitter and are thus expected

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to contain large amounts of SLs, we purified SLs from bolted crisp-head lettuce stems. The purification of SLs from frozen lettuce stems was contracted to Nagara Science Co. (Gifu). Lactucopicrin (> 95% purity) and LO (～85% purity) were purified according to the method described by Sessa et al. (2000). The purity of these SLs was confirmed by means of high-performance liquid chromatography (HPLC) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy.

Acetonitrile used for HPLC was HPLC grade, and the other reagents used were special grade and were used without further purification.

2 Materials and Sample Preparation

Endive (*Cichorium endivia*), a bitter leafy vegetable that contains lactucopicrin (Price et al., 1990, Arakawa et al., 2008), was used to determine the sample preparation and analysis conditions. In addition to endive and crisp-head lettuce, butter-head lettuce was also used to consider the preparation methods because sample preparation was expected to be easier because the structure of the heads is simple. The latex exuded from stems of crisp-head lettuce was also collected for analysis because the latex from lettuce is extremely bitter (Soma, 2006) and was expected to be rich in SLs. Crisp-head lettuces 'Falcon' (Sakata Seed) and 'Cisco' (Takii), butter-head lettuce 'Summer Green' (Sakata Seed), and endive (Takii) cultivated at the experimental field at the National Institute of Vegetable and Tea Science (Tsu, Mie) were harvested for the research from April to June of 2008 and 2009. Special care was taken at the harvest to dig the plants without cutting the main root, so that the compounds in the latex could be analyzed. The SLs of the harvested lettuce were analyzed within four hours. Heads of several other cultivars of crisp-head lettuce were also obtained from farms managed by a private company (Ibaraki Prefecture).

For the analysis of latex, about 10 μl of latex was collected from the cut surface of the base of the stem of the lettuce head. The latex was dissolved in 1 ml of methanol (containing 0.1% phosphoric acid), and the supernatant after centrifugation was analyzed as described by Sessa et al. (2000).

Lettuce leaves, which had been previously peeled from the head and rinsed in running water for 30 s, were blended in water for three min using a blender (Hamilton Beach). The extract was filtered through a No. 5 B filter (Advantec) and then passed through a 0.45-μm membrane filter (DISMIC-13 CP, Advantec); the filtrate was used for high-performance liquid chromatography (HPLC) analysis. The preparation of the leaves took less than 10 min. All the sample preparations were performed at room temperature.

3 HPLC conditions

An LC-10Avp HPLC system (Shimadzu) with high-pressure gradient pumps and a diode array detector was used. The volume of sample injected was 20 μl. A reverse-phase column was used (Mightysil RP-18 GP, 5 μm, 4.6 mm×150 mm, Kanto Chemical). Gradient elution was performed with solution A, composed of 100 mM sodium phosphate buffer (pH 2.1), and solution B, composed of 90% acetonitrile, delivered at a flow rate of 1.0 ml/min as follows (% solvent B): 24% (0-2 min), 24-36% (linearly, 2-22 min), and 36% (22-25 min). The absorbance at 262 nm was monitored (wavelength range 200-340 nm). The temperature of the column was maintained at 35℃. This set of conditions is referred to as condition 1.

For the purpose of comparing our chromatograms with those reported by Sessa et al. (2000), we slightly modified condition 1. The same column was used, but the solvent gradient was changed from 99:1 (A:B) to 48:52 (A:B) over 60 min at 1 ml/min, where A = 100 mM sodium phosphate buffer (pH 2.1) and B = 90% acetonitrile. The detection wavelength was set at 200 nm, according to the literature (Sessa et al., 2000). This set of conditions is referred to as condition 2.

Ⅲ Results and Discussion

1 HPLC analysis of lactucopicrins

With our HPLC system, we had difficulty reproducing the chromatograms reported by Sessa et al. (2000) for the analysis of LO and other SLs. Sessa et al. used 0.1% phosphoric acid as mobile phase A, but we suspected that our difficulty reproducing the chromatograms was due to the instability of the pH of the mobile phase. Therefore, we used a phosphate buffer (pH 2.1) as mobile phase A instead of 0.1% phosphoric acid, and this change improved the reproducibility.

The chromatogram of the latex of the crisp-head lettuce (obtained under condition 2) is shown in Fig. 1. The major peak (peak 1) was identified as LO by comparison with the spectrum of an LO standard. A small peak for lactucopicrin (peak 2) was also detected. We believe that some of the other peaks in the chromatogram correspond to SLs, on the basis of comparison of this chromatogram with that obtained by Sessa et al. (2000). We did not identify these minor peaks, because purifying the minor SLs was difficult. Only the amounts of LO (the major SL) and lactucopicrin (its degradation compound) were analyzed in this report.

The HPLC gradient conditions were further modified to improve the separation and shorten the analysis time for LO and lactucopicrin (condition 1), and the resulting chromatogram is shown in Fig. 2. The change in the HPLC conditions reduced the sample throughput time from > 60 min to 30 min. This method was used for analysis of the extracts of the lettuce leaves.

If an analytical method for the SLs in lettuce is to be widely useful, the difficulty in obtaining the HPLC standards will have to be overcome. Under our modified HPLC conditions, the peaks for LO and lactucopicrin were well separated from the peaks originating from the sample matrix, so estimating the retention times of LO and lactucopicrin from the chromatograms of latexes of lettuce can be expected to be easy for HPLC systems different from the one we used in this study.

For the quantification of LO and lactucopicrin, we referred to the report of Price et al. (1990), who used santonin as an internal standard for the HPLC analysis of sesquiterpenes. They determined that the ratio of the peak areas of santonin and lactucopicrin was 1.00 when the absorbance was monitored at 262 nm. Therefore, after we confirmed that (peak area)/(concentration, mg/l) for santonin was equal to the value for lactucopicrin, we used santonin as an external standard for HPLC analysis, and the amounts of lactucopicrin and LO were calculated from the ratio of the peak areas detected at 262 nm, assuming that the molecular absorption coefficients (at 262 nm) of LO and lactucopicrin were equal.

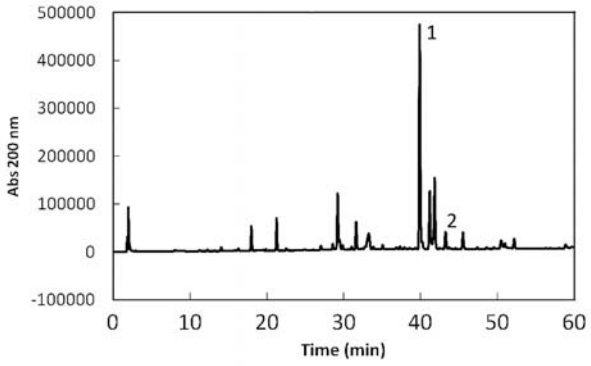

Fig. 1 Chromatogram of the latex collected from crisp-head lettuce, 'Falcon'.

1: lactucopicrin 15-oxalate (LO), 2: lactucopicrin. The chromatogram was obtained under condition 2 (described in Materials and Methods), which is a slight modification of the conditions reported by Sessa et al. (2000).

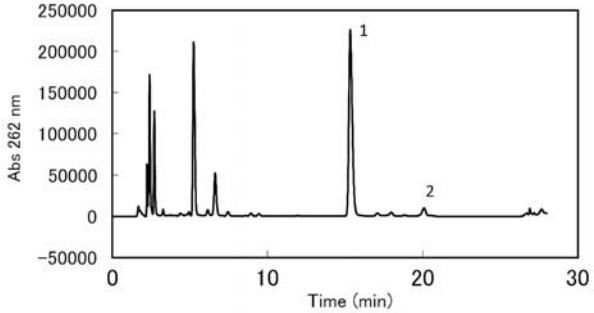

Fig. 2 Chromatogram of the latex collected from crisp-head lettuce, 'Falcon'.

1: LO, 2: lactucopicrin. The chromatogram was obtained under condition 1 (described in Materials and Methods).

2. Stability of lactucopicrin 15-oxalate and sample preparation from lettuce leaves

The LO standard solution dissolved in methanol was periodically analyzed by HPLC. Even though the sample was kept at 10℃, the peak area for LO decreased by about 2% every hour, and the peak area for lactucopicrin increased (data not shown). Because of the instability of LO, a rapid preparation method is required to prevent LO degradation.

Endive (*Cichorium endivia*) was used for the development of the sample preparation method, because it is bitter and reported to contain higher amounts of lactucopicrin than does lettuce (Price et al., 1990, Arakawa et al., 2008). Moreover the amount of latex exuded at the cut surface of the endive leaves was much lower than the amount from lettuce, and this fact is expected to increase the reproducibility of the results of the experiment. A whole endive plant previously cut from the roots was divided longitudinally into two equal parts with a cooking knife. One piece was extracted with water, and the other with ethanol. The peak areas of LO and lactucopicrin in the HPLC chromatograms were compared for the two solvents (Table 1). For both solvents, the LO content was much higher than the lactucopicrin content. The total amounts of LO and lactucopicrin were the same for the two solvents, which means that no further studies to choose the most suitable organic solvents were required. Because water extraction was faster and less expensive, leaves were extracted with water in the following experiments. LO and lactucopicrin are referred to as lactucopicrins (Ls) in the following discussion.

Arakawa et al. (2008) have already reported the amounts of SLs in *Lactuca* and *Cichorium* (including endive) species. They detected lactucopicrin in these species but did not mention the presence of LO. In our study, we found that LO was the major lactucopicrin species in endive (Table 1) and in the latex of lettuce (Figs. 1 and 2). We also detected LO as a major constituent of the Ls in the leaves of all the analyzed cultivars (crisp-head lettuces 'Falcon', 'Cisco', 'Trigger', 'Asahina', 'Prano', 'Kamogawa 12', 'Joy Green 54', 'Top Galant'; butter-head lettuce 'Summer Green'; and endive) under our conditions (data not shown). As mentioned above, LO is unstable in methanol. Arakawa et al. (2007) extracted Ls from plants using hot methanol; therefore, although LO is thought to be the major SLs in plants, it probably decomposed during the preparation steps and thus its degradation compound (lactucopicrin) was measured in their study (Arakawa et al., 2007, 2008, 2009). Because our conditions for sample preparations were much milder and more rapid than theirs, our conditions are more suitable for analyzing LO in plants.

3 Localization of lactucopicrins in lettuce

The amounts of Ls in the various parts of the crisp-head lettuce cultivar 'Falcon' are shown in Table 2. Latex collected from the cut stem contained >1% Ls (40 mmol/kg fresh weight [FW]). The stem contained ～20 μmol/kg FW. The leaves that are usually served in salad contained less than 2 μmol/kg FW of Ls on 14 May, and the amount of Ls gradually increased to 4.3 μmol/kg FW as the date of harvest grew later (that is, as the head aged). We also compared the amounts of Ls in the basal (thick) and tip (thin) parts of the same leaf (Table 3) and found that the basal part was richer in Ls than tip.

Lettuce stems are more bitter than leaves in the head, the basal (thick) part of the leaf is more bitter than the tip (thin) of the same leaf, and late-harvested lettuce is bitter (Soma, 2006); and endive is much more bitter than lettuce (Shiratori & Itaki, 2009). These observations are consistent with the amounts of Ls measured in this study, if we assume that organoleptic bitterness is related to the amount of Ls.

4 Sample preparation for analysis of lactucopicrins and organoleptic tests

The amount of Ls was extremely high in latex from crisp-head lettuce (Table 2). We expected that the amount of latex remaining in the leaves would be influenced by the sample preparation method, and that the amount of latex remaining in the leaves would affect the amount of Ls in the leaves and the organoleptic bitterness of

the leaves.

A head of butter-head lettuce 'Summer Green' was divided in two longitudinally. Half the head was immediately prepared for the analysis of Ls. The remaining half was kept at room temperature for 30 min, at which time the cut surface of the head was covered with latex. After the latex was wiped off, the half head was prepared for analysis. Comparison of the amounts of Ls in the two half heads indicated that wiping significantly reduced the amount of Ls (Table 4).

Usually lettuce leaves are well washed for organoleptic tests, whereas the leaves are not washed well for chemical analysis, to avoid the loss of the components. To determine the effects of washing, we divided butter-head lettuce 'Summer Green' equally into two parts with a cooking-knife and then tore off all the leaves of one half head and washed them well in running tap water for 30 s. The leaves of the remaining half were also torn off, but not washed in water. The amounts of Ls in the leaves were compared for the washed and unwashed leaves (Table 5). The amounts of Ls in the washed samples were always lower than the amounts in unwashed samples from the same head. These results indicate that washing reduced the amount of latex in the leaves.

The results shown in Tables 4 and 5 indicate that the analytical data for Ls was affected by how the samples were prepared. Because latex, which contains extremely large amounts of Ls, is easily lost during preparation of the leaves, sample preparation must be done carefully. Moreover, the standard deviations for the data in Tables 2-5 were relatively large in spite of the careful sample preparation, which means the amounts of Ls varied widely among individual heads of the same cultivars.

The palatabilities of various cultivars were compared organoleptically by the Committee for Investigation of the Palatability of Vegetables (Horie, 2008). Each sample was composed of a mixture of leaves from three heads of the same cultivar. Twenty panelists evaluated the palatability of each cultivar, and they ranked bitterness of the leaves from 0 (not bitter) to 3 (extremely bitter). Unexpectedly, the bitterness scores for the same cultivar ranged from 0 to 3 among the panelists in several cultivars tested (Horie, 2008). This fact suggests that the bitter compounds were heterogeneously distributed in the samples for the organoleptic tests. Heterogeneity

Table 1 Amounts of lactucopicrins in endive leaves extracted with water or ethanol

	μmoles/kg FW	
solvent	LO	lactucopicrin
water	111 ± 10	9 ± 1
ethanol	117 ± 16	3 ± 1

n=6, FW: fresh weight, LO: lactucopicrin 15-oxalate

Table 2 Amounts of lactucopicrins in parts of crisp-head lettuce and variation with date of harvest

	harvest day	Ls (μmoles/kg FW)
leaves	14-May	1.9 ± 0.5
	19-May	3.3 ± 0.8
	26-May	4.3 ± 0.9
latex	14-May	40726.8 ± 7795.1
stem	19-May	20.3 ± 6.5

n = 5, Ls: lactucopicrins (LO and lactucopicrin)
Culivar: Falcon

Table 3 Amounts of lactucopicrins in different parts of the leaves of crisp-head lettuce

	Ls (μmoles/kg FW)
basal	3.6 ± 0.6
tip	1.2 ± 0.3

n=3, $p < 0.01$ (paired t-test)
Cultivar: Falcon

Table 4 Effect of wiping off the letex exudad at the cut surface of butter-head lettuce

	Ls (μmoles/kg FW)
control	82.7 ± 13.9
wiped*	36.6 ± 10.2

n=3, $p < 0.05$ (paired t-test)
Freshly dug heads of 'Summer Green' was used.
*Latex exuded on the cut surface was wiped off.

Table 5 Effects of water-washing on the amounts of lactucopicrins in the leaves of butter-head lettuce

	Ls (μmoles/kg FW)
control (no-wash)	15.1 ± 6.9
water washing*	9.2 ± 3.3

n = 5, $p < 0.05$ (paired t-test), Cultivar: Summer Green
* Torn leaves were rinsed in running tap water for 30 s.

within a sample can be explained by the large standard deveations of the amounts of Ls in the heads (Table 3) and among the heads subjected to the same treatments (Tables 2-5). Although organoleptic tests are popular and effective for evaluating the palatability of food, supplying homogenous samples to the panelists for the evaluation of bitterness of lettuce is difficult because of the heterogeneous distribution of bitter compounds.

In conclusion, we established a simple and rapid preparation method for the analysis of Ls in lettuce leaves, and we improved sample throughput and compound separation by modifying the HPLC conditions. The presence of LO in lettuce cultivars was confirmed by means of this method. For this research, we assumed that Ls are related to the organoleptic bitterness of lettuce, and this assumption was not inconsistent with our experimental results. However, getting consistent results from organoleptic tests for bitterness was difficult because of the heterogeneous distribution of Ls within a head and between heads. The possibility remains that minor SLs or other components may contribute to the bitterness of lettuce. Moreover the intensity of bitterness is expected to be different for LO and lactucopicrin. Further research is required to clarify the details of the bitterness of lettuce.

Summary

Objective methods for evaluating the bitterness of lettuce are necessary because bitterness is an important factor for the palatability of lettuce. Some sesquiterpene lactones (SLs) are known to be bitter, and the major SL in lettuce is reported to be lactucopicrin 15-oxalate (LO). Lactucopicrin is also known to be present in lettuce. Simple sample preparation methods and conditions for the rapid analysis of LO and lactucopicrin in lettuce heads were developed. Because LO was unstable in solution, rapid sample preparation was required. Lettuce leaves were extracted with water in a blender, and the filtrate was analyzed. Sample preparation took less than 10 min. LO and lactucopicrins (referred to as Ls) could be separated by means of gradient reverse-phase high-performance liquid chromatography with pH 2.1 phosphate buffer and 90% acetonitrile as mobile phases. The sample throughput time was 30 min. In crisp-head lettuce, the amounts of Ls in latex, stems, and leaves were ～40,000, ～20, and $< 5\,\mu$mol/kg FW, respectively. Because the analytical results obtained were consistent with the strength of bitterness, we suggest that the amounts of Ls can be used as an indicator of bitterness. Because the amounts of Ls varied among heads, with head age, among the various parts of the plant, and with washing treatment, special care is needed for evaluation of bitterness by means of organoleptic tests or instrumental analysis.

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高速液体クロマトグラフィーによるレタス中のラクチュコピクリン類（苦味物質）の分析

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摘　要

レタスのおいしさに関係して苦味が重要な要素であるため，レタスの苦味を客観評価する方法の開発が必要である．セスキテルペンラクトン類（SLs）は苦いことが知られているが，レタスにおける主要なセスキテルペンラクトン類はシュウ酸ラクチュコピクリン（LO）と報告されている．さらに，ラクチュコピクリンもレタスに含まれることが知られている．そこで，レタスの結球部に含まれるLOとラクチュコピクリンについて，簡単な試料調製法と迅速な分析法を開発した．LOは溶液中で非常に不安定なことが明らかになったので，迅速な試料調製が求められる．レタスの葉をブレンダーを用いて水抽出し，濾液を分析に供した．本操作は10分以内で完了した．pH 2.1のリン酸緩衝液と90%アセトニトリルを移動相とするグラジエント逆相高速液体クロマトグラフ法により，2種類のラクチュコピクリン類（Ls）の分離が可能であった．なお，分析時間は30分であった．レタスにおいてLsの含量は，湿重量1 kgあたり，乳液，芯，葉でそれぞれ40000，20マイクロモルおよび5マイクロモル以下であった．得られた分析結果が感覚的な苦味に一致したので，Ls含量を苦味の指標として用いる可能性が示唆された．ただし，Lsの含量は個体，熟度，部位，洗浄処理により異なるため，官能評価や機器分析により苦味を評価する際には注意が必要である．

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