Formation by Mechanical Stimulus of the Flavor Compounds in Young Leaves of Japanese Pepper (*Xanthoxylum piperitum* DC.)

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The volatile compounds formed in slapped and crushed young leaves of Japanese pepper (*Xanthoxylum piperitum* DC.) were compared with those of intact leaves by using a dynamic headspace gas analysis combined with GC-MS in an on-line system, together with the results of a sensory evaluation. The results indicated that the factors influencing the aroma note were mainly the constituent oxygenated monoterpenes and C_6 compounds and the quantity of terpene hydrocarbons. To clarify the formation mechanism for the aroma, the activities of lipoxygenase and β-D-glucosidase were investigated. The results revealed that the hydrolysis of glycosides and the degradation of unsaturated fatty acids both played an important role in the formation of the major aroma compounds in young leaves of Japanese pepper.

Keywords: *Japanese pepper; Xanthoxylum piperitum DC.; flavor; formation; oxygenated monoterpenes; C_6 compounds*

INTRODUCTION

Japanese pepper (*Xanthoxylum piperitum* DC.) is a plant native to the Japanese islands, mainland China, and the Korean peninsula. Fresh young leaves and dried fruits of Japanese pepper are used as a spice to impart a fresh flavor or to suppress any unpleasant fishy and meaty odor in dishes. Sakai et al. (*1*) and Kusumoto et al. (*2*) have analyzed the composition of the aroma and found >100 components from the essential oil of the young leaves and dried fruits, with citronellal being considered to be the most important contributor to the characteristic aroma among the constituents. The aroma concentrates of green fruits and young leaves adsorbed on Porapak Q resin have recently been analyzed and quantified by Wu et al. (*3*). Moreover, the aroma compounds prepared by steam distillation under reduced pressure have been evaluated by an aroma extract dilution analysis (AEDA) in our laboratory (*4*), and citronellal and citronellol were found to be the characteristic aroma attributes.

In Japan, young leaves of Japanese pepper are commonly utilized in Japanese dishes after being directly slapped with the hands or crushed in a mortar. Although intact leaves revealed almost no aroma, the characteristic aroma was released by such mechanical stimuli. The characteristic aroma of slapped leaves is also different from that of crushed leaves. We were interested in both the aroma composition causing the intense odor and the formation mechanism, but no chemical data were available on the slapped or crushed leaves, nor were any details about the formation mechanism.

It has recently been shown that odorless and nonvolatile glycosides can be hydrolyzed by an acid or enzyme to liberate volatile aroma compounds, and various glycosidically bound volatiles have been detected in almost 50 plant families as an important potential source of aroma (*5*). The activity of β-D-glucosidase, an important hydrolase, has been investigated, and some enzymes have been purified and characterized in tea leaves and wines. Kojima et al. (*4*) have reported that some alcoholic aroma compounds were liberated from young leaves of Japanese pepper by a crude enzyme in the acetone powder, which is the acetone insoluble fraction obtained from fresh leaves.

On the other hand, the green odor is responsible for the flavor of the plants, especially with young leaves. The formation of C_6 compounds responsible for the green odor has also been proved to be derived from enzymatic oxidation of linolenic acid and linoleic acid in the disrupted tissues of leaves (*6*), virgin olive oil (*7*), and tomato (*8*). It is known that lipoxygenase, hydroperoxide lyase, alcohol dehydrogenase, and alcohol acetyltransferase play an important role in these processes. Additionally, the glycoside of (*Z*)-3-hexenol as a green aroma precursor has been isolated from tea leaves (*9*, *10*). Two possible pathways have consequently been confirmed for the formation of the green odor.

Our aim in the present study is to identify the differences in the aroma compounds and in the formation mechanism in fresh young leaves when they are subjected to two different mechanical stimuli simulating the treatment used for cooking. We analyzed the volatile composition and quantified the main compounds by the dynamic headspace method combined with GC-MS in an on-line system. We also investigated the action of lipoxygenase and glucosidase in an attempt to clarify the formation mechanism for the main aroma.

MATERIALS AND METHODS

Material. Fresh young leaves of Japanese pepper that had been cultivated in Saitama prefecture in Japan were purchased from a local market in the early part of May 1999. Part of the sample for the sensory evaluation and GC-MS analysis was refrigerated at 4 °C, and the remainder of the sample for preparing acetone powder was frozen at −80 °C until needed.

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10.1021/jf001166m CCC: $20.00
Published on Web 02/10/2001

Chemicals. α-Pinene (>98%), (*Z*)-3-hexenol (98%), citronellol (>98%), limonene (98%), citronellal (98%), α-phellandrene (98%), linalool (98%), and citronellyl acetate (98%) were obtained from Kanto Chemical Ltd. (Tokyo, Japan); linolenic acid (99%) and linoleic acid (99%) were from Sigma-Aldrich (Tokyo, Japan). Polyclar AT, which was used to remove polyphenols, was purchased from Gokyo Industries, Ltd. (Osaka, Japan).

Analysis of the Volatile Composition. *Sample Preparation.* To obtain the same characteristic odor as that of food dishes, samples were damaged with two mechanical stimuli. Young leaves (0.5 g) as fresh as possible were placed flat on a cork pad, and a steel plate of 2-kg weight (surface area = 15 cm × 15 cm) was dropped onto them three times from a height of 17 cm to simulate slapping with the hands. Crushed leaves (0.5 g) were obtained by grinding in mortar for 30 s. Intact young leaves were also analyzed as a control. Just after preparation, each sample was taken for both a sensory evaluation and GC-MS analysis.

Sensory Evaluation. The sensory evaluation was performed on the same day that the sample had been purchased. The test panel consisted of nine assessors who were trained in describing with common words the flavor profile of different concentrations of authentic chemicals. The authentic chemicals used were presumed to be the main components in young leaves of Japanese pepper. After the samples had been prepared as already described and transferred to a beaker (∅ 6.0 cm × 5.8 cm), they were immediately presented to each assessor for a pretest. Fifteen descriptive words were collected from the assessors, and seven common words from them were selected for the sensory evaluation. The prepared samples were presented in a random sequence to the assessors, and they were asked to rate the score for each flavor intensity by using a scale of 0 (absent) to 4 (strong). The resulting data were evaluated by an analysis of variance (ANOVA).

Gas Chromatographic Analysis. The GC-MS analysis was carried out by the dynamic headspace analysis method with an on-line multipurpose-sampling thermal desorption system (MSTD-258, GL Sciences Ltd.). A Hewlett-Packard (HP) 5890 series II gas chromatograph coupled with an HP 5972 mass spectrometer was used, in which a DB-Wax capillary column (J&W, 60 m × 0.25 mm i.d.) was connected to the GC instrument. The analytical conditions were as follows: helium carrier gas flow rate, 1.0 mL/min; injector temperature, 220 °C; oven temperature program, 40 °C rising to 220 °C at a rate of 2 °C/min. The MS instrument was operated in the EI mode with an ionization voltage of 70 eV.

A prepared sample (0.5 g) was put into a small dish shaped like a watch glass (∅ 7.4 cm × 0.7 cm) and set in a quartz chamber (∅ 20 cm × 4.0 cm) in the oven. After being purged for 5 min at room temperature, the headspace gas generated from the sample was collected for adsorbing on Tenax TA (100 mg) in a tube by passing helium (50 mL/min of flow) for 15 min at 32 °C. The headspace volatiles were then desorbed by rapidly heating the tube to 270 °C and simultaneously concentrated in the injector cryofocusing tube of the GC-MS instrument at −130 °C for 10 min by a thermal desorption cold trap injector (TCT), before being finally injected into the GC column at 270 °C in the splitless mode.

Quantification of the Main Aroma Compounds. A quantitative analysis was performed based upon a series of calibration curves for authentic chemicals. The authentic chemicals used were α-pinene, α-phellandrene, *d*-limonene, (*Z*)-3-hexenol, citronellal, linalool, citronellyl acetate, and citronellol (0.5 mg of each) and were dissolved together in a 50-mL flask with methanol. One milliliter of each of the resulting solutions was sealed in ampules and stored at −28 °C until being diluted to 5, 10, 20, 100, or 200 times, respectively. In the case of the authentic samples, each solution was completely adsorbed on 96 mg of Tenax TA in a laboratory dish (∅ 2.8 cm × 1.4 cm) set in the chamber. The MSTD system and GC-MS instrument were operated as previously described. The conditions were also similar, except that purging was for 0.1 min, desorbing the sample was at an oven temperature of 200 °C, and collection in the Tenax TA tube was for 20 min.

The transformation ratio of citronellal under the conditions used in this study was investigated, because it was observed that citronellal was cyclized by a simple thermal process, as reported in the literature (*11*). Under our analytical conditions, it was found that ∼79% of citronellal was converted to isopulegol, which has several isomers. The calibration curve for citronellal was consequently obtained according to this transformation ratio.

The total ion chromatogram (TIC) peak area data were plotted against the concentration of the authentic chemical, so that the volatile compounds could be quantified with reference to the calibration curves.

Assay of Enzymatic Activity. *Lipoxygenase.* The presence of lipoxygenase was investigated by modifying the method of Sekiya et al. (*12*). The young leaves (1.0 g) were immersed in 14 mL of McIlvaine buffer (32 mM citric acid/135 mM Na_2HPO_4, pH 6.3) and homogenized by a mixer under nitrogen at 4 °C for 2 min. The supernatant was used as a crude lipoxygenase solution after the homogenate had been filtered through four layers of gauze.

Immediately after the crude enzyme solution had been prepared, a 1-mL aliquot in a 10-mL vial sealed with a rubber stopper was preincubated at 35 °C for 1 min. A 13.2-μmol amount of linolenic acid (or linoleic acid) as a substrate and methyl decanoate as an internal standard were then added, and the mixture was shaken vigorously at 25 °C for 1 min and incubated at 35 °C for 10 min. During the incubation, the headspace gas was collected by a solid-phase microextractor [SPME, with a 100 μm poly(dimethylsiloxane) coating for the 5-7330 holder], and the C_6 aldehydes that had been formed were analyzed by GC. The GC analysis used the same column as those described above and was operated isothermally at 100 °C; the injector temperature was 200 °C, and the detector (FID) temperature was 220 °C. Under these conditions, the retention times of hexanal, (*Z*)-3-hexenal, and (*E*)-2 hexenal were 9.0, 10.1, and 12.6 min, respectively. The reaction without the substrate was used as a blank.

Measurement of the Glucosidase Activity. The method used for preparing the acetone powder was similar to that reported by Kojima et al. (*4*). Fresh young leaves (450 g) frozen at − 80 °C were dipped in acetone (3 L) that had been cooled to −50 °C. The acetone solution was removed by filtration after the leaves had been homogenized for 3 min in a dispersing mixer. This process was repeated eight times. The residue was then completely dried under reduced pressure, the yield of acetone powder being 37.8 g, which was stored at −80 °C until needed.

The activity of glucosidase was determined by hydrolyzing *p*-nitrophenyl glucoside (*p*-NPG) according to the method of Agrawal and Bahl (*13*). Acetone powder (1 g) and Polyclar AT (0.5 g) were dissolved in a 20 mL of a citrate buffer (50 mM at pH 5.0). The suspension was homogenized at 10000*g* for 30 s with a blender under cooling at 0 °C. Then after two centrifugations at 13500*g* for 20 min at 4 °C, the resulting supernatant was used as the crude enzyme solution. The activity of glucosidase for hydrolyzing *p*-NPG controlled at 37 °C was determined by a UV spectrometer at 420 nm. The boiled supernatant was used as a blank.

RESULTS AND DISCUSSION

Sensory Evaluation. The fresh young leaves of Japanese pepper, after being slapped by the hands or crushed in a mortar, are generally used as a seasoning for Japanese dishes. The slapped leaves are usually used to impart a pleasant flavor, whereas the crushed leaves are often added to miso to prepare a seasoning sauce. Their flavors are quite different. To evaluate the difference in aroma characteristics produced by these two mechanical stimuli, we conducted a sensory evaluation with the result presented in Figure 1. The aroma intensity of the intact leaves was the weakest, giving the lowest score among the three samples. The 3.5 score for a pleasant flavor was highest with the slapped

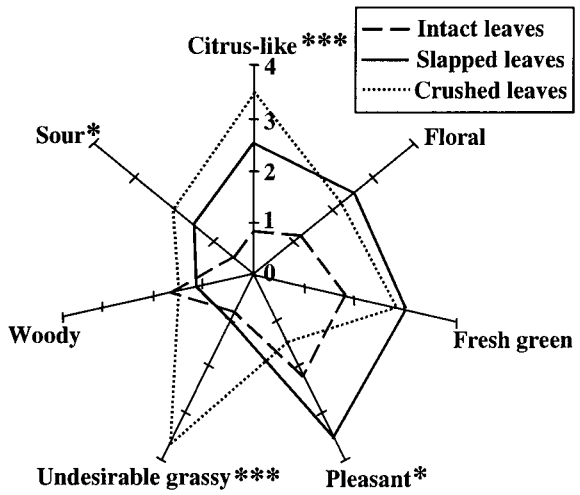

Figure 1. Flavor profiles of the young leaves of Japanese pepper prepared by different mechanical stimuli. Each value represents the average score on a scale of 0 (absent) to 4 (strong). *, $p < 0.05$; ***, $p < 0.001$.

leaves, which might have been due to the high scores for citrus-like (2.5), floral (2.5), and fresh green (3.0) odors. The undesirable grassy aroma of the crushed leaves was too strong (3.7) and obviously reduced the pleasant flavor (1.5), although high scores for citrus-like (3.5), fresh green (2.8), and floral (2.2) odors were obtained. It was apparent that the citrus-like odor significantly increased after the young leaves had been injured by a mechanical stimulus, although there was little difference in the scores for floral, fresh grassy, and woody odors among the samples. However, the scores for the undesirable grassy and pleasant odors were significantly different between the slapped and crushed leaves.

Differences in the Composition and Quantity of Volatiles. The volatile compounds formed were analyzed by using dynamic headspace gas sampling coupled with a GC-MS analysis to evaluate the flavor difference between the mechanically damaged leaves. These compounds were identified by comparison with the mass spectra and the Kovats indices (KI) of those of authentic chemicals. The gas chromatograms are shown in Figure 2, and the compounds detected are listed in Table 1. The number of compounds detected in the leaves was increased by the mechanical stimuli, being 12 (intact leaves), 22 (slapped leaves), and 36 (crushed leaves). In particular, because the quantities of terpene hydrocarbons, C_6 compounds, and oxygenated monoterpenes were markedly different, the quantity of each was measured by using a calibration curve and is summarized in Table 1.

In the slapped leaves, α-pinene (2.71 μg/g), *d*-limonene (0.56 μg/g), β-phellandrene (0.41 μg/g), and caryophyllene (0.39 μg/g) were the main components of the terpene hydrocarbon fraction, the quantity of each being markedly higher at 200−1000% than that in the intact leaves. Although most of the terpene hydrocarbons do not impart strong aroma characteristics, it seems that the large quantity could have affected the flavor of the samples. *d*-Limonene in particular is considered to contribute to the characteristic aroma as the base note from a study on characterizing citrus aroma quality by odor threshold values (*14*). (*Z*)-3-Hexenol (0.63 μg/g), which is called a green alcohol, was the most predominant component at 84% of the total C_6 compounds,

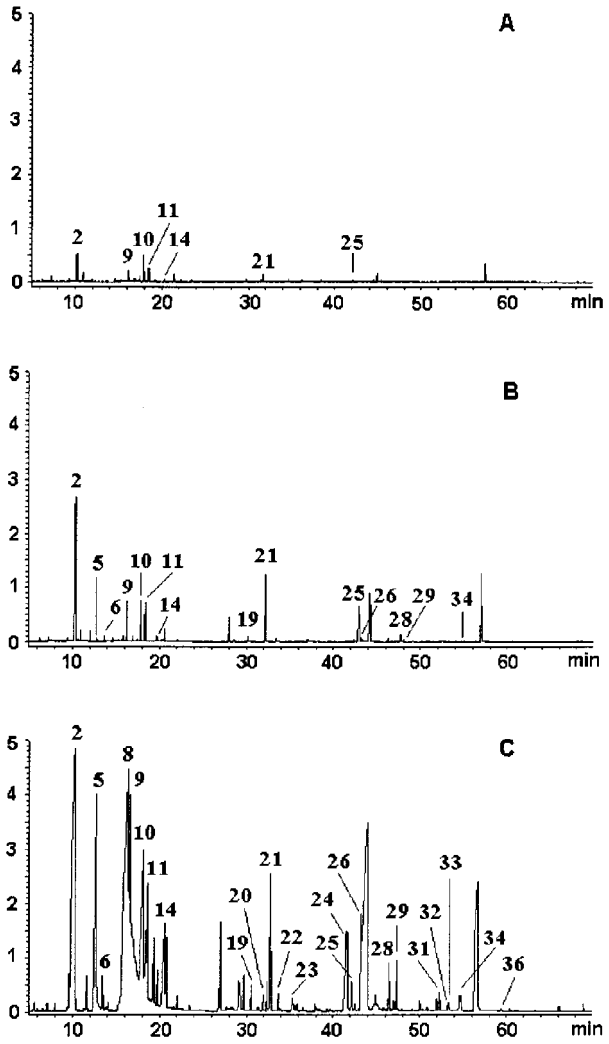

Figure 2. Comparison of the headspace gas chromatograms of the volatile components from young leaves of Japanese pepper: (A) intact leaves; (B) slapped leaves; (C) crushed leaves. The vertical axis represents the abundance of peaks; the peak numbers correspond to those in Table 1.

whereas (*E*)-2-hexenal (0.03 μg/g), which is called a green aldehyde, was present at only 3%. Although the odor threshold value of (*Z*)-3-hexenol is a little lower than that of (*E*)-2-hexenal (*15*), it is evident that the fresh green odor came mostly from (*Z*)-3-hexenol in the slapped leaves because of the high amount present. Additionally, the oxygenated monoterpenes, isopulegol (0.04 μg/g), citronellyl acetate (0.04 μg/g), and citronellol (0.02 μg/g), were identified. Citronellal, which has been considered to be the main contributor to the characteristic flavor of Japanese pepper, was not detected, although the trapping time was extended to 30 min (data not shown). In trying to quantify citronellal, we calculated the transformed ratio of citronellal to isopulegol as 79%. If citronellal has been present, we ought to have obtained ~0.01 μg/g of citronellal from 0.04 μg/g of isopulegol that was detected in the slapped leaves. However, we could not detect even a trace of citronellal. This is probably a valid result, but it is possible that the quantity of citronellal was too small to detect with our analytical instrument. Devos et al. (*16*) have reported that the odor threshold value of citronellol was a little lower than that of citronellal, and Kojima et al. (*4*) have indicated by a sniffing test that the odor of citronellol was sweeter than that of citronellal. We

Table 1. Volatile Compounds Identified and Quantified by GC-MS in the Headspace Gas from Young Leaves of Japanese Pepper

peak	volatile compound[a]	KI[b]	intact leaves	slapped leaves	crushed leaves
1	1-penten-3-one*	1038			*d*
2	α-pinene	1046	0.20 ± 0.06[c]	2.71 ± 0.92	19.61 ± 3.74
3	α-fenchene*	1082		*d*	*d*
4	camphene*	1089	*d*	*d*	*d*
5	hexanal	1112		0.05 ± 0.04	6.77 ± 0.26
6	*β*-pinene	1126		0.03 ± 0.00	0.51 ± 0.04
7	4-thujene*	1138			*d*
8	(*Z*)-3-hexenal	1180			17.88 ± 1.33
9	*β*-myrcene	1184	0.02 ± 0.00	0.08 ± 0.08	6.37 ± 1.60
10	*d*-limonene	1210	0.20 ± 0.08	0.56 ± 0.04	8.37 ± 2.13
11	*β*-phellandrene	1218	0.20 ± 0.06	0.41 ± 0.04	6.41 ± 1.89
12	(*Z*)-*β*-ocimene*	1234			*d*
13	*γ*-terpinene*	1240		*d*	*d*
14	(*E*)-2-hexenal	1247	0.02 ± 0.00	0.03 ± 0.02	2.56 ± 0.26
15	(*E*)-*β*-ocimene*	1251	*d*	*d*	*d*
16	terpinolene*	1278		*d*	*d*
17	2,6-dimethyl-2,4,6-octatriene*	1286	*d*	*d*	*d*
18	(*Z*)-2-pentenol*	1365			*d*
19	1-hexanol	1386		0.03 ± 0.00	0.27 ± 0.05
20	(*E*,*E*)-2,4-hexadienal	1407			0.62 ± 0.08
21	(*Z*)-3-hexenol	1418	0.08 ± 0.02	0.63 ± 0.13	3.03 ± 0.58
22	(*E*)-2-hexenol	1433			0.16 ± 0.02
23	citronellal	1469			1.02 ± 0.12
24	linalool	1559			0.60 ± 0.12
25	caryophyllene	1574	0.02 ± 0.00	0.39 ± 0.35	3.88 ± 1.55
26	isopulegol	1587		0.04 ± 0.00	0.73 ± 0.25
27	2-undecanone*	1601	*d*	*d*	*d*
28	humulene	1652		0.06 ± 0.02	0.60 ± 0.18
29	citronellyl acetate	1660		0.04 ± 0.00	0.48 ± 0.06
30	α-terpineol	1729			0.03 ± 0.01
31	piperitone	1738			0.16 ± 0.07
32	geranial	1749			0.02 ± 0.01
33	geranyl acetate	1760			0.03 ± 0.01
34	citronellol	1782		0.02 ± 0.00	0.10 ± 0.04
35	2-tridecanone*	1828	*d*	*d*	*d*
36	geraniol	1866			0.02 ± 0.02

[a] The volatile compounds are listed in order of their retention times. An asterisk indicates that the compound was not quantified. [b] Kovats index on DB-Wax. [c] Average concentration ± deviation (*μ*g/g of sample); $n = 3$. [d] Detected.

therefore presumed that the pleasant aroma was contributed by a good balance of the green note of the C_6 compounds, the rose-like odor of citronellol and citronellyl acetate, and the minty odor of isopulegol (*17*).

In the crushed leaves, the quantity of terpene hydrocarbons, C_6 compounds, and oxygenated monoterpenes was much greater than that in intact leaves. α-Pinene (19.61 *μ*g/g), *d*-limonene (8.37 *μ*g/g), *β*-phellandrene (6.41 *μ*g/g), and *β*-myrcene (6.37 *μ*g/g) were still the main components of the terpene hydrocarbons, but a large number of C_6 aldehydes and oxygenated terpenes were also detected. (*Z*)-3-Hexenal (17.88 *μ*g/g), which was completely absent in the slapped leaves, and hexanal (6.77 *μ*g/g) were produced at dramatically higher ratios of 57 and 22%, respectively, of total C_6 compounds, whereas (*Z*)-3-hexenol (3.03 *μ*g/g) and (*E*)-2-hexenal (2.56 *μ*g/g) also increased sharply. It is known the C_6 aldehydes have low odor threshold values among the C_6 compounds (*15*), so we considered that the undesirable grassy aroma was mainly due to the excessive quantity of C_6 aldehydes in the crushed leaves. With respect to the oxygenated monoterpenes, citronellal (1.02 *μ*g/g), linalool (0.60 *μ*g/g), isopulegol (0.73 *μ*g/g), and citronellyl acetate (0.48 *μ*g/g) were present in predominant quantities of 32, 19, 23, and 15%, respectively, among the oxygenated monoterpenes. It was concluded that the presence of citronellal and linalool contributed to the strong lemony and floral flavor in the crushed leaves because of their low odor threshold values (*16*, *18*).

Aroma Formation. From the results of the sensory evaluation and the headspace analysis, the difference of the characteristic flavor in the slapped and crushed leaves was focused on C_6 compounds and oxygenated monoterpenes.

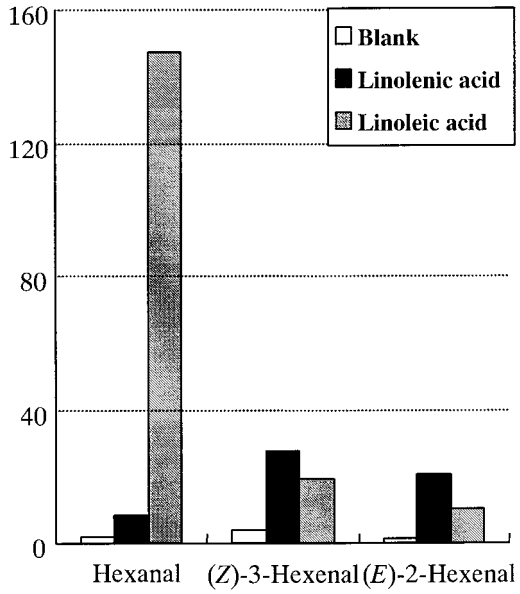

Figure 3. Influence of linolenic acid and linoleic acid in the formation of C_6 aldehydes in crushed leaves of Japanese pepper. Each value represents the ratio of the aldehyde peak area to that of the internal standard taken as 100.

Although it has been proved that C_6 compounds were

produced through linolenic acid or linoleic acid by the enzymatic reactions of lipoxygenase and lyase, there are no reports concerning lipoxygenase in young leaves of Japanese pepper. To clarify the formation of the C_6 compounds in this study, we investigated the lipoxygenase activity by GC analysis of the headspace gas of C_6 aldehydes produced when linoleic or linolenic acid was added to the leaves. The results are shown in Figure 3. Hexanal from linoleic acid and (*Z*)-3-hexenal and (*E*)-2-hexenal from linolenic acid were increased by 77, 7, and 19 times over the blank sample, respectively. It is thus obvious that lipoxygenase exists in the young leaves of Japanese pepper. Consequently, we considered that a large number of C_6 aldehydes produced in the crushed leaves were contributed by the oxidative degradation of unsaturated fatty acid, and then C_6 alcohols were mainly formed from the corresponding aldehydes by alcohol dehydrogenase or an isomerization factor (*6*).

On the other hand, in the slapped leaves, (*Z*)-3-hexenol was also identified significantly, but (*Z*)-3-hexenal was not found at all. The fact indicated that it is difficult to explain the formation of (*Z*)-3-hexenol by the same pathway with that in crushed leaves, that is to say that (*Z*)-3-hexenol was first produced in the slapped leaves before (*Z*)-3-hexenal was formed.

In the recent decade, it has been well established that flavorless glycosides represent one main accumulation form of aroma constituents in many plant materials (*5*). Many glycosides with alcohol aglycons of geraniol, linalool, α-terpineol, citronellol, and 2-phenylethanol have been isolated, and it has been determined that their alcohol aglycons can be released by endogenous glycosidases. Concerning the formation of (*Z*)-3-hexenol, Kobayashi et al. (*10*) have also pointed out that it was from enzymatic hydrolysis of its glycoside at the early stage of black tea manufacturing process. In addition, Kojima et al. (*4*) previously reported that (*Z*)-3-hexenol, citronellol, geraniol, 2-phenylethanol, and benzyl alcohol could be liberated from the glycoside-containing fraction in young leaves of Japanese pepper. Therefore, in the present experiment, we measured the *β*-D-glucosidase activity of young leaves and determined it as 5.0 units/100 g of fresh leaves (1 unit = 1 *μ*mol of *p*-nitrophenol produced/min). These results suggested that in the slapped leaves (*Z*)-3-hexenol was produced from glycoside under the action of hydrolytic enzymes.

With regard to the formation of the major alcoholic monoterpenes, citronellol, isopulegol, linalool, and geraniol, detected in slapped and crushed leaves, respectively, we presumed they were liberated from the enzymatic hydrolysis of the corresponding glycosides as described above. However, more study on the formation of these alcoholic aromas is necessary. Currently, the isolation and structural elucidation of these glycosides are being performed.

From the results of the present study, we conclude that the hydrolysis of glycosides and oxidative degradation of fatty acids play important roles in the aroma formation of young leaves of Japanese pepper for use in Japanese cuisine.

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Received for review September 22, 2000. Revised manuscript received December 26, 2000. Accepted December 29, 2000. This study was partially supported by the Yamazaki Spice Foundation.

JF001166M