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Formation Mechanism of Grassy Odor Substance in Alfalfa Seedlings

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Sensory test of the grassy odor with 2, 3, 5 and 7-day-old seedlings of alfalfa showed that 2-day-old seedlings are the grassiest. To identify the substance responsible for the grassy odor, the composition of fatty acid and the characteristics of lipoxygenase were investigated. The results showed that more than 50% of total fatty acid is linoleic acid; lipoxygenase has a high specificity to linoleic acid and produces mainly 13-*Z*, *E*-HPOD from linoleic acid. Because 13-*Z*, *E*-HPOD is converted to *n*-hexanal which has a grassy odor, the content of *n*-hexanal in the seedlings after germination was measured. It was found that it did not change remarkably throughout the tested growth period. Most of the *n*-hexanal was adsorbed weakly to insoluble materials with hydrophobic interaction. However, when the seedlings were homogenized, soluble *n*-hexanal increased rapidly and the increase was greatest in 2-day-old seedlings. Among three enzymes involved in the formation of *n*-hexanal, only the change in lipoxygenase activity correlated with that in the increase of *n*-hexanal.

Keywords: alfalfa, *n*-hexanal, grassy odor, lipoxygenase

Alfalfa (*Medicago sativa* L.) is a well known important fodder. The seedling, which germinates in dark places, is used frequently as a food material in salads, sandwiches, soups or in panfried dishes in Europe and North America. Because the seedling contains the nutritional constituents protein, lipid, sugar, vitamins A, B_1, B_2, C, E, K and U, iron, calcium and fiber in a good balance, it is believed to have high nutritive value (Levin, 1998). It is also a low-calorie healthy food. Furthermore, its ingestion is assumed to prevent diseases such as cancer, kidney trouble, heart problems, angina pectoris and osteoporosis (Quinn, 1997; Turner, 1997; Meyerowitz, 1999). These characteristics of alfalfa seedlings have aroused wide interest. In Japan the seedlings are also sold as a fresh vegetable, and in juice and tablet form, although these products are not popular primarily because of their undesirable grassy odor.

Generally, grassy odors of plants are caused by volatile C_6- and C_9-aldehydes and/or their corresponding alcohols (Hatanaka *et al.*, 1995; Fabre & Goma, 1999). Especially in soybean, *n*-hexanal known to be the main substance responsible for the undesirable odor. Even a small amount of *n*-hexanal gives off undesirable grassy odor, because of the low olfactory threshold. This makes it important to prevent the formation of *n*-hexanal in the processing of soybean products (Rackis *et al.*, 1979).

Two enzymes, lipoxygenase and hydroperoxide lyase are believed to be involved in the formation of *n*-hexanal (Matoba *et al.*, 1985a,b; Hatanaka *et al.*, 1987; Minke *et al.*, 2000). Linoleic acid is peroxidized by lipoxygenase and subsequently cleaved by hydroperoxide lyase, which leads to the formation of *n*-hexanal and ω-oxo-acids. Between these two enzymes, lipoxygenase is thought to be the key enzyme controlling the formation of *n*-hexanal. Hajika *et al.* (1991) succeeded in breeding a soybean line lacking all lipoxygenase isozymes in the seed and the use of this soybean seed made it possible to manufacture products with better taste and flavor (Furuta *et al.*, 1996; Hajika *et al.*, 1996; Nishiba *et al.*, 1995).

In alfalfa, unlike soybean the main grassy odor substance has not been identified. Identification of the substance may enable production of seedlings without the undesirable grassy odor. In this study, we found that *n*-hexanal was increased rapidly by homogenization of the seedlings and that this increase was closely related to generation of the grassy odor. It was also indicated that generation of the odor was controlled by lipoxygenase activity.

Materials and Methods

Plant material Alfalfa seeds (*Medicago sativa* L.) were soaked in water for 6 h and then germinated in the dark at 25°C. The seedlings were watered twice in a day.

Chemicals *p*-Nitrophenyl palmitate, α-linolenic acid and esculetin were purchased from Sigma Chemical. (St. Louis, MO). Acetonitrile, 2,4-dinitrophenylhydrazine and tetrahydrofuran were from Nacalai Tesque (Kyoto). Linoleic acid, hydrogen chloride-methanol Reagent 10 and nordihydroguaiaretic acid (NDGA) were from Tokyo Kasei Kogyo (Tokyo). All other reagents were chemical grade.

Sensory test A sensory test was performed to examine changes in the grassy odor of the seedlings after germination. The odor was tested with 2, 3, 5 and 7-day-old seedlings by the ranking method, in which seedlings were ranked in order of the relative intensity of the odor (1=strongest, 4=weakest). The panelists were 8 male and 12 female Japanese students of Niigata University ranging in age from 20 to 22 years. The results were calibrated using the table with S as the coefficient of concordance on the order of Kendall (Masuyama & Kobayashi, 1989).

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Fatty acid composition After lyophilization, 3-day-old seedlings were ground using a mortar and pestle. Total lipids were extracted according to the Bligh-Dyer method (Bligh & Dyer, 1959) and methylated with HCl-CH_3OH. The resulting fatty acid methyl esters were injected onto a HITACHI Model G-5000A gas chromatograph (Tokyo) coupled with a flame-ionization detector and a capillary column (TC-WAX; 60 m×0.25 mm i.d., df=0.25 μm, GL Sciences, Tokyo). The temperature conditions were as follows : column, 220°C; injector and detector, 250°C.

Enzyme assay For the assay of lipoxygenase activity, the seeds and the germinated seedlings were ground in a mortar with liquid nitrogen and further homogenized in ice-cold 50 mM phosphate buffer (pH 6.8) with a Potter-Elvejhem type homogenizer. After the homogenate had been centrifuged at 24,000×*g* for 30 min at 4°C, the supernatant was used as a crude enzyme preparation. Lipoxygenase activity was determined by the increase in absorbance at 234 nm arising from the conjugated diene in the produced hydroperoxide. The reaction mixture contained 0.15 ml of 2.5 mM linoleic acid prepared by the method of Ben-Aziz *et al.* (1970), 0.05 ml of a crude enzyme preparation and 2.80 ml of 50 mM phosphate buffer (pH 5.7), and the absorbance at 234 nm was monitored by a spectrophotometer HITACHI U-2000 at 25°C. One unit of the activity was defined as the enzyme amount producing 1 μmol of hydroperoxide per min using a molar extinction coefficient of 25,000 $M^{-1}cm^{-1}$.

For the assay of hydroperoxide lyase activity, the seedlings were homogenized as described above except that the buffer (pH 6.8) contained 0.1% (v/v) Triton X-100. After gentle stirring for 20 min in an ice bath, the homogenate was centrifuged at 14,000×*g* for 20 min at 4°C, and the supernatant was used as a crude enzyme preparation. Hydroperoxide lyase activity was determined by the decrease in absorbance at 234 nm at 25°C according to the method of Nathalie and Jan (1997). The standard reaction mixture contained 0.06 ml of 1 mM 13-hydroperoxylinoleic acid prepared by the method of Wu and Robinson (1995), 0.12 ml of a crude enzyme preparation and 2.82 ml of 50 mM phosphate buffer (pH 6.8). One unit of the activity was defined as the enzyme amount consuming 1 μmol of hydroperoxide per min.

For the assay of lipase activity, a crude enzyme preparation was obtained as described for the assay of hydroperoxide lyase activity. Lipase activity was determined by measuring the absorbance at 400 nm due to the *p*-nitrophenol released by hydrolysis of *p*-nitrophenyl palmitate with a spectrophotometer (Galliard, 1971). One unit of the activity was defined as the enzyme amount producing 1 μmol of *p*-nitrophenol per min.

Reaction products of lipoxygenase The reaction products of lipoxygenase were analyzed with linoleic acid as substrate. The crude enzyme preparation (1 unit) from 3-day-old seedlings was incubated with 0.25 mM linoleic acid in 10 ml of 0.1 M phosphate buffer (pH 5.7) for 30 min under constant aeration at room temperature. The products were extracted according to Bligh-Dyer method. The extract was dried by nitrogen gas and dissolved in 2 ml of hexane/2-propanol/acetic acid (984 : 15 : 1, v/v/v). A high performance liquid chromatograph (HPLC, HITACHI L-6300) equipped with a UV-VIS detector (HITACHI L-4250) and an integrator (HITACHI D-7500) was used to analyze the reaction products. The column used was a LiChrosorb SI 60-5(φ4.6×250 mm; GL Sciences). The products were eluted with hexane/2-propanol/acetic acid (984 : 15 : 1, v/v/v) at a flow rate of 1.0 ml/min and detected at 234 nm. Standards of 13-hydroperoxide (HPOD) and 9-HPOD were prepared using lipoxygenase from soybean (Wu & Robinson, 1995) and potato (Sekiya *et al.*, 1977), respectively.

Determination of n-hexanal *n*-Hexanal was determined as the 2,4-dinitrophenylhydrazone derivative with HPLC according to the method of Matoba *et al.* (1985a), except for the use of perchloric acid instead of phosphoric acid. The germinated seedlings were ground in a mortar with liquid nitrogen, and then homogenized in 5% perchloric acid with a homogenizer of Potter-Elvejhem type at room temperature. The homogenate was used for determination of *n*-hexanal. The HPLC equipment described above was utilized and the column was LiChrosorb RP-18-5 (φ4.6×250 mm; GL Sciences) with a protective column (φ4×10 mm; GL Sciences). The derivative was eluted with acetonitrile/water/tetrahydrofuran (75 : 24 : 1, v/v/v) at a flow rate of 1.0 ml/min and detected at 350 nm.

Results

Sensory test The grassy odor of 2, 3, 5 and 7-day-old seedlings was tested by the ranking method. Among 20 the panelists, 15 judged that 2-day-old seedlings were the grassiest (Fig. 1); most also judged that the grassy odor of 3, 5 and 7-day-old seedlings was second, third and fourth, respectively. The coefficient of concordance on the order of Kendall was calibrated using the table with S. Consequently, because of S=996>364.2 (α=1%), the evaluation of panelists was shown to be significantly in accord.

Fatty acid composition The grassy odor substances in alfalfa seedlings are assumed to be mainly C_6-, C_9-aldehydes and/or the corresponding alcohols as well as those in other plants. Because these substances are generally produced from fatty acids, the fatty acid composition of 3-day-old seedlings, which were about the same size as those sold in Japan, was

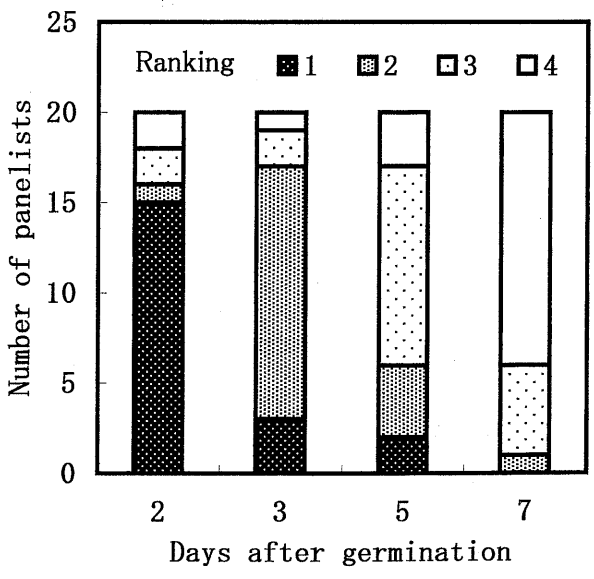

Fig. 1. Sensory test of undesirable grassy odor in alfalfa seedlings. Seedlings grown for 2, 3, 5 and 7 days were ranked in order of relative intensity of grassy odor (1=strongest; 4=weakest).

Table 1. Composition of fatty acid and substrate specificity of lipoxygenase in 3-day-old seedlings.

	Fatty acid (%)	Relative activity (%)
Palmitic acid	11.5	—
Oleic acid	11.7	—
Linoleic acid	52.7	100.0
α-Linolenic acid	24.1	46.2

—, not determined.

investigated. As shown in Table 1, the lipid of alfalfa seedlings contained palmitic acid, oleic acid, linoleic acid and α-linolenic acid, with linoleic acid comprising more than 50% of the total fatty acid.

Substrate specificity of lipoxygenase Substrate specificity of lipoxygenase from 3-day-old seedlings was evaluated using the crude enzyme preparation. Because the optimum pH of the enzyme was pH 5.7, the enzyme assay was performed at this pH. The lipoxygenase in alfalfa seedlings exhibited high activity to linoleic acid, two times higher than that to α-linolenic acid (Table 1).

Reaction products of lipoxygenase The reaction products of lipoxygenase to linoleic acid were investigated using the crude enzyme preparation of 3-day-old seedlings by straight-phase HPLC. The elution profile showed one major peak and several minor peaks (Fig. 2). The retention time of the major peak coincided with that of the standard of 13-*Z*, *E*-HPOD prepared using lipoxygenase from soybean. This result indicates that the lipoxygenase in alfalfa seedlings primarily converts linoleic acid to 13-*Z*, *E*-HPOD.

Changes in the amount of n-hexanal To reveal whether the grassy odor in alfalfa seedlings is caused by *n*-hexanal, quantitative changes in *n*-hexanal after germination were determined. The seedling content of *n*-hexanal measured by homogenizing the seedlings with 5% perchloric acid slightly decreased on day 2 and then increased gradually, but no remarkable changes were observed throughout the growth period tested (Fig. 3A). On the

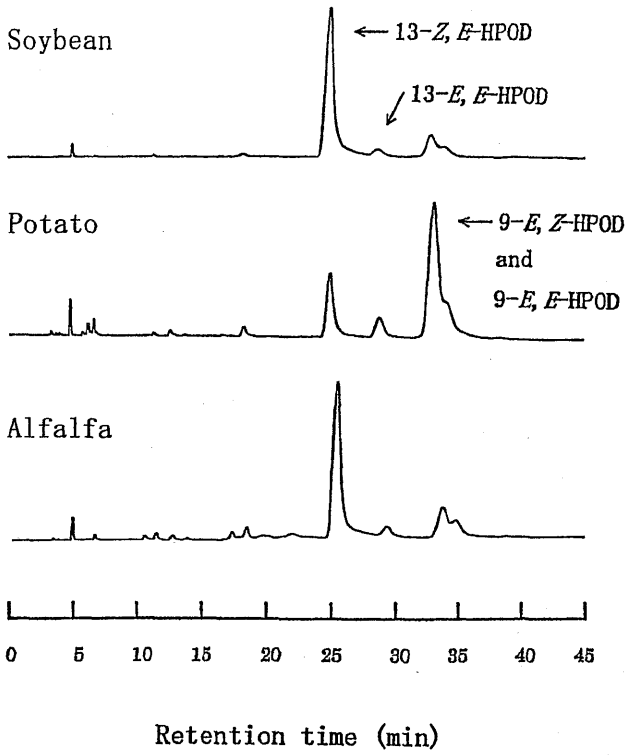

Fig. 2. HPLC analysis of linoleic acid oxygenized by lipoxygenase.

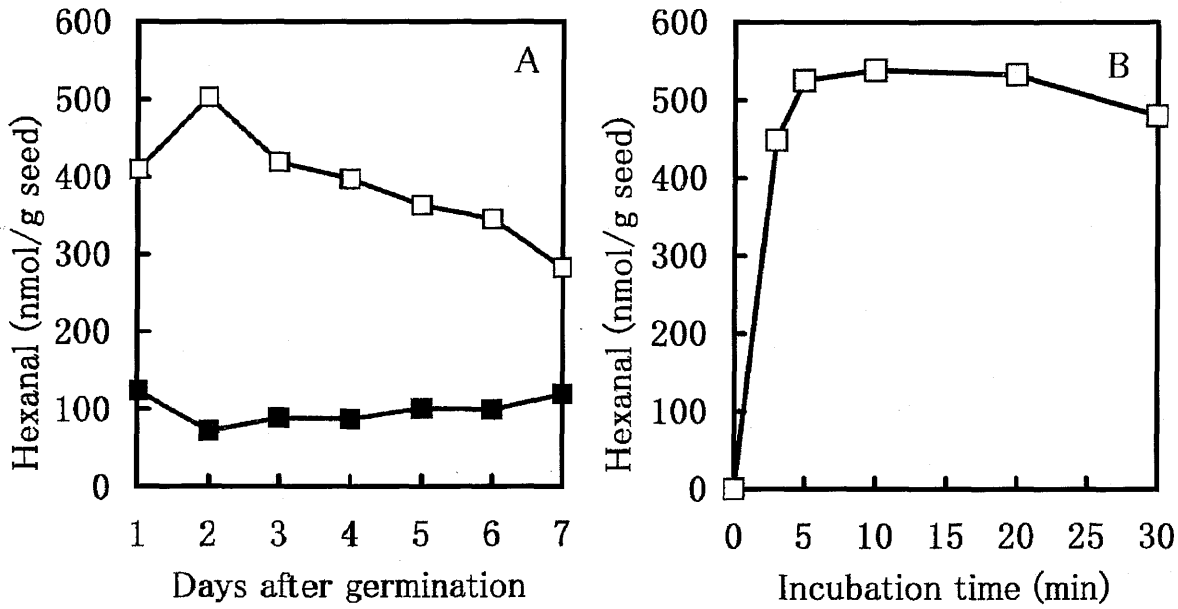

Fig. 3. (A) Quantitative changes of *n*-hexanal in alfalfa seedlings after germination. The content of *n*-hexanal (□) was measured by homogenizing the seedlings with 5% perchloric acid. The amount of *n*-hexanal increased by homogenization (■) was calculated by subtracting the content of *n*-hexanal in the seedlings from the amount understood in homogenate prepared with 50 mM phosphate buffer, pH 6.8, at room temperature. (B) Time course of increase of *n*-hexanal in the homogenate. After 3-day-old seedlings were ground in a mortar with liquid nitrogen, 50 mM phosphate buffer (pH 6.8) was added and the amount of *n*-hexanal was examined during the incubation at 37°C.

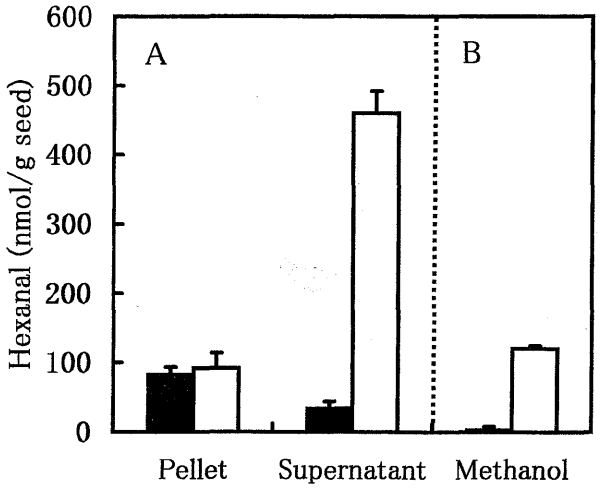

Fig. 4. Solubility of *n*-hexanal. (A) After 3-day-old seedlings were ground in a mortar with liquid nitrogen and then homogenized in 50 mM phosphate buffer (pH 6.8) with (■) or without 5 mM $SnCl_2$ (□), the homogenates were centrifuged at 24,000×*g* for 30 min. Amounts of *n*-hexanal in the pellet and the supernatant were measured. (B) The pellet prepared by the addition of $SnCl_2$ was resuspended in methanol, and then recentrifuged at 24,000×*g* for 30 min. Amounts of *n*-hexanal in the pellet and the supernatant were measured. ■, pellet; □, supernatant.

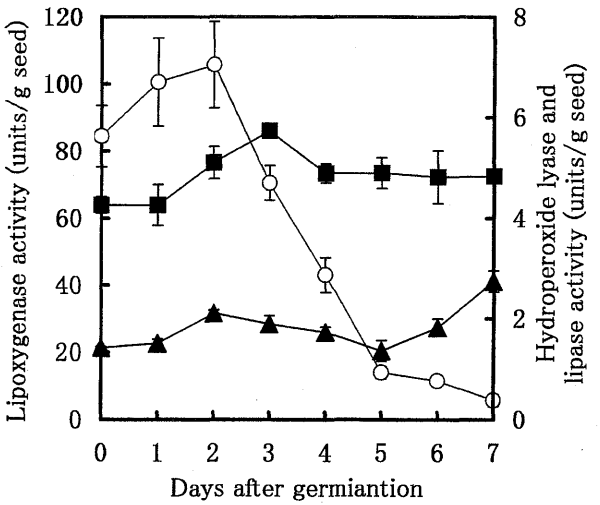

Fig. 5. Changes in lipoxygenase (○), hydroperoxide lyase (■) and lipase (▲) activities of the seeds and seedlings after germination.

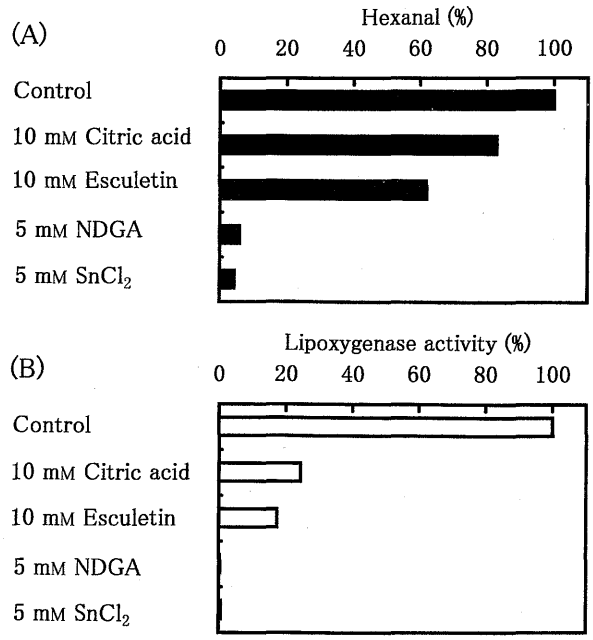

Fig. 6. Effects of lipoxygenase inhibitors on *n*-hexanal formation and lipoxygenase activity. (A) After 3-day-old seedlings were homogenized with each lipoxygenase inhibitor, the amount of *n*-hexanal in the homogenate was measured. The homogenate without the inhibitors was used as a control. The amount of *n*-hexanal increased by homogenization was calculated by subtracting the content of *n*-hexanal in the seedlings from the amount of *n*-hexanal in homogenate prepared with 50 mM phosphate buffer, pH 6.8, at room temperature. (B) After 3-day-old seedlings were homogenized with 50 mM phosphate buffer (pH 6.8), and the supernatant was centrifuged and used as a crude enzyme preparation. Lipoxygenase inhibitor was added to the reaction mixture and the lipoxygenase activity was measured by spectrophotometer for citric acid and NDGA and by oxygen electrode (Kuribayashi *et al.*, 2002) for esculetin and $SnCl_2$. The reaction without the inhibitors was used as a control.

other hand, when the seedlings were homogenized with 50 mM phosphate buffer (pH 6.8) at room temperature, the amount of *n*-hexanal in the homogenate remarkably increased in comparison with the content in the seedlings (Fig. 3A). This increase was greatest on day 2 and thereafter gradually decreased. When the time course of *n*-hexanal increase in the homogenate of 3-day-old seedlings was investigated, rapid increase was shown for up to 5 min, where it reached a plateau (Fig. 3B).

Solubility of n-hexanal To determine whether *n*-hexanal in the seedlings exists in the soluble or insoluble fraction, the homogenate of 3-day-old seedlings was centrifuged and the amounts of *n*-hexanal in the pellet and the supernatant were measured. When the seedlings were homogenized with a lipoxygenase inhibitor, $SnCl_2$, these amounts were 84 and 35 nmol/g seed, respectively (Fig. 4A). This result suggests that a considerable amount of *n*-hexanal in the intact seedlings is in the insoluble fraction and is adsorbed to insoluble materials. In contrast, when the seedlings were homogenized without the inhibitor, only the amount of *n*-hexanal in the supernatant increased up to 13 times than that in supernatant prepared with the inhibitor. The pellet prepared with the inhibitor was resuspended in methanol and centrifuged, and the resultant supernatant contained most of *n*-hexanal (Fig. 4B).

Changes in activities of lipoxygenase, hydroperoxide lyase and lipase Time course of activities of the three enzymes in the seeds and seedlings after germination were measured. Lipoxygenase activity increased up to day 2, and then remarkably decreased (Fig. 5). Hydroperoxide lyase activity was highest on day 3, but slightly declined on day 4 and then remained constant. Lipase activity slightly increased up to day 2, then decreased gradually and increased again from day 5.

Effects of lipoxygenase inhibitors on the formation of n-hexanal The roles of lipoxygenase in the formation of *n*-hexanal were determined by homogenizing 3-day-old seedlings in buffer containing citric acid, esculetin, NDGA or $SnCl_2$, and then measuring the amount of *n*-hexanal in the homogenate. The formation of *n*-hexanal was inhibited by addition of the inhibitors; however, there were large differences in the effect on the formation of *n*-hexanal among the tested inhibitors: from 83% in citric acid to 4% in $SnCl_2$ (Fig. 6A). In evaluating the effects of these

inhibitors on lipoxygenase activity in the 3-day-old seedlings, NDGA and $SnCl_2$ were found to inhibit most of the activity. In citric acid and esculetin, 24 and 17% of the activity remained, respectively (Fig. 6B). This indicates that the remaining activity in the homogenate participated in the formation of *n*-hexanal.

Discussion

The purposes of this study were to identify the main substance responsible for the grassy odor substance in alfalfa seedlings and to determine when and how it is formed.

The grassy odor substances in plants are known to be volatile C_6- and C_9-aldehydes and/or their corresponding alcohols (Hatanaka, 1995; Fabre & Goma, 1999). In particular C_6- and C_9-aldehydes are contained in many plants, *e.g.*, cucumber (Galliard & Phillips, 1976), tomato (Riley *et al.*, 1996), olive (Jose *et al.*, 1993) and bell pepper (Matsui *et al.*, 1997) and they contribute to the characteristic flavors of each plant. However, these substances also cause serious problems in the food processing of legumes. Because these aldehydes are generally formed via peroxidation of fatty acids by lipoxygenase, the composition of fatty acids and the characteristics of lipoxygenase in alfalfa seedlings were of interest. The results showed that more than 50% of total fatty acid is linoleic acid and the lipoxygenase is highly specific to linoleic acid. Analysis of the reaction products revealed that the lipoxygenase produces mainly 13-*Z*, *E*-HPOD from linoleic acid. Minke *et al.* (1999) reported that hydroperoxide lyase activity in alfalfa seedlings was two times higher to 13-HPOD than to 9-HPOD. 13-HPOD is degraded by hydroperoxide lyase and converted into *n*-hexanal. These suggest that *n*-hexanal causes the grassy odor in alfalfa seedlings.

To confirm this idea, the content of *n*-hexanal in the seedlings after germination was measured; it was found to remain almost constant throughout the tested growth period. The quantitative changes in *n*-hexanal after germination were quite different from results of the sensory test, in which the grassy odor was strong in the early stage of growth and then decreased gradually. Jose *et al.* (1993) reported that the formation of C_6-aldehydes in plants is related to the destruction of cells. For example, homogenization of plant tissues increases C_6-aldehydes very quickly through enzymatic reactions in the presence of oxygen (Schreier, 1981; Tressl *et al.*, 1983). When alfalfa seedlings were homogenized in 50 mM phosphate buffer (pH 6.8) at room temperature, the amount of *n*-hexanal in the homogenate increased remarkably and quickly. This increase was greatest on day 2 and the quantitative changes after germination agreed with results of the sensory test. The pH of homogenate of the seedlings coincides with that of saliva (pH 6.4–7.0). Therefore, the undesirable grassy odor may be caused by the increase in *n*-hexanal through the destruction of seedlings by mastication.

Hexenal, which is formed from α-linolenic acid, also was presumed to be related to the undesirable grassy odor of alfalfa seedlings, because the olfactory threshold value (0.11 ppm) is lower than the 0.35 ppm of *n*-hexanal (Rackis *et al.*, 1979). When the seedlings were homogenized, not only *n*-hexanal but also hexenal increased, however, the increase of the latter was much less (Sekiya *et al.*, 1979) and the quantitative changes of hexenal after germination did not agree with results of the sensory test (data not shown). These suggest that not hexenal but *n*-hexanal is more closely related to the grassy odor.

In the intact seedlings, *n*-hexanal concentration was about 1.5 ppm throughout the tested growth period and was larger than 0.35 ppm, the olfactory threshold value of *n*-hexanal (Rackis *et al.*, 1979). However, more than 70% of *n*-hexanal adsorbed to insoluble materials. Because *n*-hexanal was released by methanol, the adsorption is presumed to be hydrophobic. It is unclear whether the adsorbed *n*-hexanal is eluted by masticating the seedlings, but *n*-hexanal in the intact seedling is believed not to be appreciably concerned in the undesirable grassy odor, because the amount increased by the homogenization was much larger than that in the intact seedlings.

n-Hexanal is synthesized from free linoleic acid by reaction of two enzymes, *i.e.*, lipoxygenase and hydroperoxide lyase. Because linoleic acid is released by the action of lipase from glycerolipid, phospholipid and so on, it is assumed that lipase is also related to the formation of *n*-hexanal. Among these three enzymes, only the change of lipoxygenase activity after germination relatively agreed with that of the amount of *n*-hexanal increased by homogenization. This is evidence that the formation of *n*-hexanal is controlled strongly by lipoxygenase activity. To confirm that lipoxygenase controls the formation of *n*-hexanal, the seedlings were homogenized in the presence of lipoxygenase inhibitors and the amount of *n*-hexanal was measured. The formation of *n*-hexanal was depressed by the addition of the inhibitors, but because the depression by citric acid and esculetin was unremarkable, effects of the inhibitors on lipoxygenase activity were investigated. The activity was found to be inhibited strongly by NDGA or $SnCl_2$, but not remarkably inhibited by citric acid or esculetin. The degree of depression on the formation of *n*-hexanal by the inhibitors was correlated with that of inhibition of enzyme activity. These results demonstrate that the formation of *n*-hexanal is controlled by lipoxygenase activity.

Thus, it is presumed possible to remove the grassy odor by repression of this activity. Various methods of repression have been reported: acid treatment, heating, alcohol treatment, microwave irradiation, and soaking in alkali (Lee & Lillard, 1997). However, these methods are not only expensive and imperfect, but also difficult to apply for alfalfa seedlings, because they are mainly consumed as a raw vegetable. Therefore, the genetic elimination of lipoxygenase may be the most effective approach to reduce undesirable grassy odor in alfalfa seedlings. Some lipoxygenase isozymes are generally contained in plant seedlings, and their characterization will be required to identify the isozyme, which is involved in the formation of *n*-hexanal in alfalfa seedlings.

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