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Geraniol Dehydrogenase Activity Related to Aroma Formation in Ginger (*Zingiber officinale* Roscoe)

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Geranial is one of the potent odor compounds in fresh ginger. To clarify the generation of geranial in ginger, the alcohol dehydrogenase activity was measured in a crude enzymatic system of ginger. This enzyme solution was found to contain geraniol dehydrogenase (GeDH) specifically acting on geraniol as a substrate with NADP as a coenzyme. Geranial generation and GeDH activity were investigated for different maturity stages and storage periods of ginger. Both were at maximum levels from just after harvesting to initial storage. The GeDH activity subsequently dropped, and the generation of geranial also stopped. These results suggest that the GeDH activity in ginger is related to the generation of geranial.

Keywords: *Ginger (Zingiber officinale Roscoe); geraniol dehydrogenase; geranial; flavor*

INTRODUCTION

The characteristic citrus and pleasantly fresh aroma of ginger has prompted its use as a spice and food ingredient all over the world. The aroma profile of ginger and the composition of the volatile compounds have been differently reported, depending on the place of harvest (*1−7*). Many studies related to food distribution and processing have reported the influence on the composition of volatile compounds by the extraction process, γ-irradiation, and drying and cooking of ginger (*8−12*). The characteristics and composition of the aroma therefore seemed to be important factors in these studies to determine the quality of ginger. However, despite these studies on the volatile composition, there are few data on aroma formation in ginger.

Among the volatile compounds in fresh ginger, Nishimura (*13*) has reported that the potent aroma compounds were mainly such monoterpenoids as geraniol, linalool, and geranial by an aroma extract dilution analysis. In order to increase the pleasant aroma of ginger, it is therefore necessary to obtain information about the formation mechanisms for these monoterpenoids. We have previously isolated the glucosides or disaccharides of such potent aroma monoterpene alcohols as geraniol, nerol, linalool, citronellol, and 2-heptanol and suggested that these glycosides were the aroma precursors or intermediates in fresh ginger (*14*). In addition to these monoterpene alcohols, geranial, which presents a citrus-like aroma, is both organoleptically and quantitatively one of the main aroma compounds in ginger. Geranial has recently received attention for its antimicrobial, antitumor promotion, and antidepressant effects and as an inhibitor of retinoic acid biosynthesis (*15−19*). Therefore, the content of geranial is valuable not only for improving the ginger flavor but also for enhancing its physiological functions. With respect to the formation of geranial in ginger, Sakamura (*20*) has reported that the geranial content in an aroma extract of ginger tended to increase during storage, in contrast to the decrease in contents of geraniol and geranyl acetate. Enzymatic factors could therefore be expected to be related to the oxidation of geraniol to geranial. The action of alcohol oxidase (AOX) and alcohol dehydrogenase (ADH) has been reported to influence the enzymatic oxidation of monoterpene alcohols in some herbs and spices (*21−25*). ADH, in particular, is one of the most common biologically active enzymes. Therefore, we examined in this present work the enzymatic activity of ADH and AOX in relation to the geranial formation in ginger, their characteristics being investigated in a crude enzymatic system prepared from the acetone powder of ginger.

Fresh immature or mature ginger is preferred in Japanese dishes to dried ginger. These types of ginger are usually used in quite different ways from each other because of their different flavors and textures. Because mature ginger just after harvest in the autumn is stored in a cold room at a controlled temperature and humidity in Japan, mature ginger is available on the market throughout the year. Mature ginger has a stronger citrus aroma than that of the immature plant, so the change in ginger aroma during storage seems to be connected to the generation of geranial. We therefore also investigated the relationship between the quantity of geranial formed and the ADH activity during the growth and storage of ginger to clarify the mechanism of increase in content of geranial in ginger.

MATERIALS AND METHODS

Plant Material. Rhizomes of two varieties of ginger (*Tosaichi* and *Kogane-no-sato*; *Zingiber officinale* Roscoe) were purchased directly from the cultivating company (Sakata Nobuo Shoten, Kochi, Japan). These rhizomes were cultivated in the same field, and immature ginger was harvested 70 days earlier than usual. The remainder was harvested at the usual

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time, and storage samples were prepared by keeping them at under 15 °C wrapped in plastic according to the normal commercial procedure. After careful washing with distilled water, each rhizome was subjected to the experiments. Acetone powder from each sample was prepared by following the procedure described in a previous paper (*14*).

Chemicals. Alcohols as substrates were purchased from Tokyo Chemical Industry (Tokyo, Japan), citral and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and NADP and peroxidase (from horseradish) were obtained from Boehringer Mannheim (Germany).

Assay for ADH Activity. A crude enzyme solution was prepared from the acetone powder of ginger. Each acetone powder sample was homogenized twice for 30 s with a Tris-HCl buffer (pH 7.2, 4 °C) containing 15% glycerol, 2 mM dithioerythritol, and 10 mM mercaptoethanol. The slurry was centrifuged at 22500*g* (20 min × 2, 4 °C), and the resulting supernatant was used in the assay for ADH activity after being appropriately diluted.

1. Measurement of the Change from NAD/NADP to NADH/NADPH. The measurement of ADH activity was examined according to a modification of the procedure of Sangwan et al. (*23*). The enzyme solution was prepared from 1 g of acetone powder with 25 mL of the buffer by using the foregoing procedure.

Geraniol was used as a substrate after being dissolved in DMSO. Geraniol (2 mM) and NAD or NADP (0.65 mM) were mixed in 3.5 mL of a 0.1 M glycine−NaOH buffer (pH 9.0). The reaction was started by adding 0.5 mL of the crude enzyme solution, and the mixture was incubated for 11 min at 25 °C. The change in absorbance at 340 nm, indicating the reduction of NAD or NADP, was measured for 10 min, and the change in quantity was converted to that of generated citral. To investigate the influence of different alcohols as the substrate, eight alcohols, nerol, citronellol, borneol, menthol, benzyl alcohol, heptanol, 2-heptanol, and ethanol, were each incubated in the reaction mixture instead of geraniol. A control was prepared by adding DMSO instead of each alcohol.

2. Measurement of Generated Citral. A crude enzyme solution was prepared from 2 g of acetone powder with 25 mL of the above-mentioned buffer. One milliliter of the crude enzyme solution was added to 3 mL of a 0.1 M Tris-HCl buffer containing 2 mM geraniol and 0.65 mM NAD or NADP. This solution (pH 9.0) was incubated at 25 °C for 1, 2, 6, or 16 h, and the enzyme reaction was stopped in an ice bath. After 1.0 mg of methyl decanoate had been added as an internal standard, the generated geranial and remaining geraniol were extracted twice with diethyl ether (1 mL) and dried with anhydrous Na_2SO_4. After concentration, this extract was subjected to a GC analysis. A control was prepared by using a boiled enzyme solution.

AOX Activity for Geraniol. Geraniol oxidase activity was measured according to the method of Banthorpe et al. (*24*). The reaction mixture contained 3 mL of a 0.1 M Tris-HCl buffer (pH 9.0), 1.65 mM geraniol (added as a DMSO solution), 25 units of peroxidase, *O*-dianisidine (200 μg), and the enzyme solution prepared as already described. The reaction mixture was incubated at 25 °C for 10 min, and the reaction was stopped by adding 0.2 mL of 4.0 M HCl. The color change was spectrophotometrically measured at 400 nm.

Quantification of Geraniol-Related Compounds in Ginger. Thirty grams of ginger at each stage was homogenized with 40 mL of diethyl ether. Methyl decanoate (0.5 mg) was added as an internal standard, and the ethereal layer was separated by centrifugation. After two more extractions with diethyl ether, the resulting extracts were combined as the fraction including volatile compounds. The ethereal solution was dried overnight with anhydrous Na_2SO_4, and the aroma concentrate was obtained by distilling off the ether at 39.5 °C. To remove such hydrocarbons as sesquiterpenes, this ethereal extract was subjected to silica gel column chromatography and separated into pentane and pentane/ether (1:1) fractions. The pentane/ether (1:1) fraction, which included geranial and other oxygen-containing compounds, was subjected to a GC analysis.

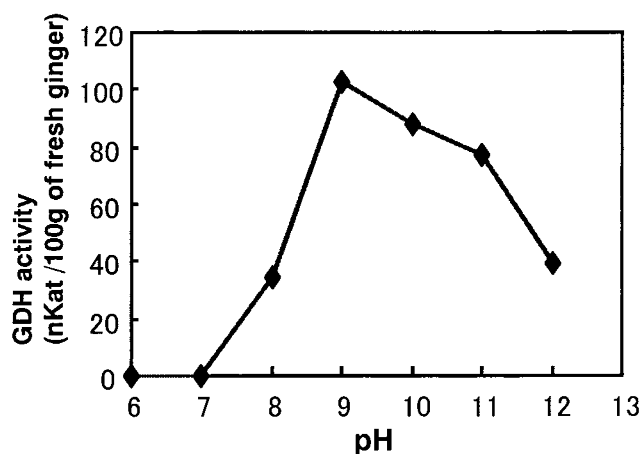


Figure 1. pH dependence of the GeDH activity in ginger. One nanokatal indicates 1 nmol/s of NADP reduction.

This procedure was conducted in triplicate. The amounts of geranial, geraniol, and geranyl acetate were calculated from calibration curves produced from each authentic compound.

GC Analysis. A Hewlett-Packard 5890 series II gas chromatograph equipped with an FID was used for this analysis with a DB-Wax (60 m × 0.25 mm i.d., J&W) fused silica capillary column. The oven temperature was initially held at 60 °C for 4 min and then raised to 200 °C at 2 °C/min. The injector and detector temperatures were set at 200 and 220 °C, respectively, and helium was used at 1.0 mL/min as the carrier gas. Each compound was identified by comparing its retention time and mass spectrum with those of the authentic compound.

RESULTS AND DISCUSSION

ADH Activity for Geraniol in Ginger. To clarify the relationship between geraniol and geranial in ginger, the ADH and AOX activities were investigated in the crude enzyme system of ginger. The acetone powder prepared from mature ginger var. *Tosaichi* was used for this. The optimum pH value for the oxidation of geraniol to geranial was first investigated, and the results are summarized in Figure 1. The activity was very stable at more than pH 8.0 and maximum at pH 9.0. On the other hand, it was found that the enzyme lost activity at pH $\leq$7.0. Therefore, the ADH activity was measured at pH 9.0. Figure 2 shows gas chromatograms of the aroma extracts from the enzyme reaction mixture containing geraniol with either NAD or NADP as the coenzyme, after incubation at pH 9.0 for 16 h. The addition of NAD resulted in no peak being detected other than those of the substrate, geraniol, and a trace of a geranial derivative from the crude enzyme solution of ginger. On the other hand, such new peaks as citral (geranial and neral) were detected after geraniol had been incubated with NADP in the enzyme solution. The quantities of geranial and neral after 1, 2, 6, and 16 h of incubating the reaction solution with NADP are shown in Table 1. Each value was calculated by the ratio of the peak area and response factor compared to those of the internal standard by the GC analysis. The formation of geranial and neral was observed from the addition of NADP, their quantities increasing with longer incubation time. The total amounts generated after 16 h of incubation were about 9.5 times those after 1 h of incubation. The ratio of generated neral to geranial was always in the range of 2:3−1:2 in the experiments, indicating that geranial was predominantly generated over neral. These results suggest that the

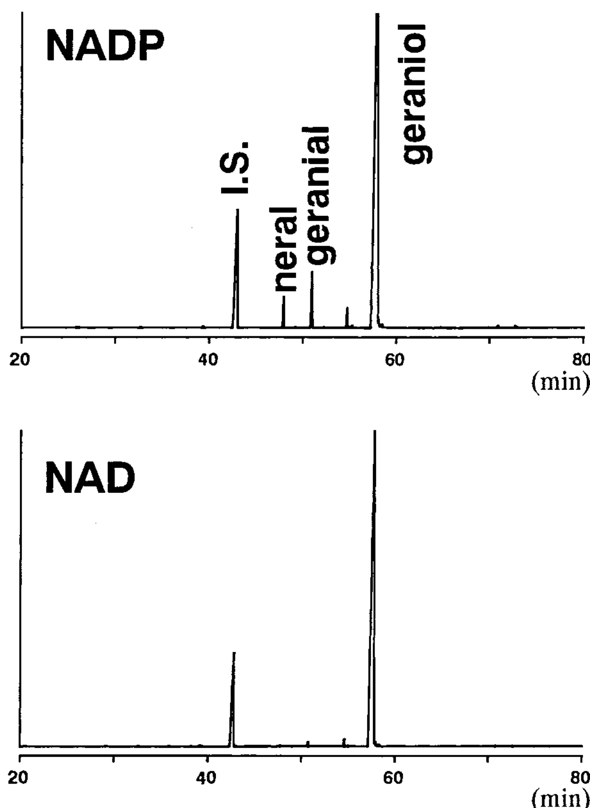


Figure 2. Gas chromatograms of the volatile compounds after incubation of geraniol in a crude enzyme solution with added NADP or NAD. The internal standard (I.S.) was methyl decanoate.

Table 1. Generation of Geranial and Neral by Incubation of Geraniol with NADP in a Crude Enzyme Solution of Ginger[a]

incubation time (h)	geranial (mg)	neral (mg)	citral (mg)
1	0.050	0.031	0.081
2	0.073	0.052	0.125
6	0.210	0.175	0.385
16	0.487	0.280	0.767

[a] Values were calculated according to the ratio of the peak area to that of the internal standard (1.0 mg of methyl decanoate) and each response factor.

rhizomes of ginger contained alcohol dehydrogenase depending not on NAD but on NADP for geraniol as the substrate. On the other hand, no AOX activity was apparent in the enzyme solution from ginger. Therefore, ADH, but not the oxidase, is suggested to have played an important role in the oxidation of geraniol in ginger rhizomes.

The ADH activity with various alcohols as the substrate was measured, and the results are shown in Table 2. The four terpene alcohols, two aliphatic alcohols, and one phenolic alcohol, nerol, citronellol, borneol, menthol, heptanol, 2-heptanol, benzyl alcohol (2 mM), and ethanol (20 mM), were investigated as substrates instead of geraniol. Among these alcohols, geraniol, nerol, citronellol, borneol, and 2-heptanol were identified in the aroma extract of ginger. The relative activity for each alcohol was calculated as a percentage of the activity for geraniol. The activity for geraniol was the strongest, those for nerol and citronellol were, respectively, 52.9 and 20.6% that of geraniol, and 2-heptanol and heptanol had lower activity. A GC analysis of the

Table 2. ADH Activity for Various Alcohols

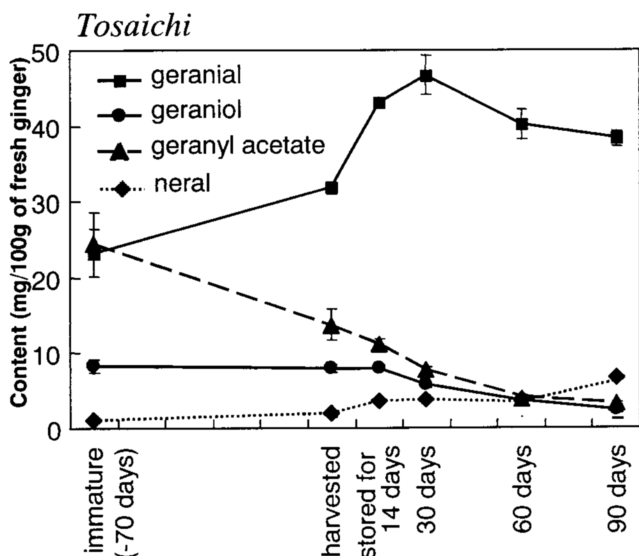
substrate	nkat/100 g of fresh ginger	relative activity[a]
geraniol	48.8	100.0
nerol	26.3	52.9
citronellol	9.4	20.6
borneol	0.0	0.0
menthol	0.0	0.0
benzyl alcohol	0.0	0.0
heptanol	7.5	7.4
2-heptanol	9.4	10.3
ethanol	0.0	0.0

[a] The relative activity is presented as a percentage of that for geraniol.

enzyme reaction solution with nerol indicated that both geranial and neral were generated. In the case of citronellol, citronellal was detected by GC. The activity for each of the other alcohols was very weak, this enzyme not showing any activity for borneol, menthol, benzyl alcohol, and ethanol. These results suggest that the enzyme of ginger had unique specificity for some compounds. Because geraniol, nerol, and citronellol are structurally similar acyclic monoterpene alcohols, ADH in ginger must be geraniol dehydrogenase (GeDH), which specifically acts on geraniol. The geranial reduction activity by this enzyme solution was also measured as 14.0 nkat at pH 6.0. Because the ADH activity depends on the pH value, concentration of the substrate, and ratio of NADP to NADPH, it was difficult to define the priority for oxidation or reduction. However, geraniol is the most fundamental alcohol in the biosynthesis of terpenoids, and GeDH in orange juice has been reported to be unstable under acidic conditions (*25*); therefore, GeDH is presumed to have acted for oxidation under the basic conditions in the plant.

With respect to the oxidation of geraniol to geranial in plant materials, Banthorpe et al. have purified AOX that oxidized geraniol from *Tanacetum vulgare* (*24*). On the other hand, Sangwan et al. (*23*) have partially purified GeDH from lemongrass. They reported that this catalyzed geraniol by an NADP-dependent action and, moreover, determined its molecular weight as 84 kDa. Potty and Bruemmer (*25*) have also partially isolated GeDH from orange fruit (orange juice), which specifically acted on geraniol with NADP as a coenzyme. The foregoing results indicate that the GeDH property in ginger could be similar to that in both lemongrass and orange. However, this is the first report of GeDH activity being detected in ginger and in part of the rhizome of the plant material. The rhizome of ginger might therefore have a peculiar aroma formation mechanism.

Relationship between Geranial Generation and Geraniol Dehydrogenase Activity during the Growth and Storage of Ginger. To investigate whether the geranial generation in ginger during storage was connected to the activity of GeDH or not, the amount of each and activity in ginger were measured at various growth stages and times of storage. Two varieties of ginger (*Z. officinale* Roscoe) cultivated in Japan, *Tosaichi* and *Kogane-no-sato*, were harvested from the same field and used for the experiments. Aroma extracts and acetone powder samples were obtained from immature (harvested 70 days earlier than usual), mature (just after harvest at the normal time), and stored (15, 30, 60, and 90 days after harvest at the normal time) ginger. Each preparation was tested



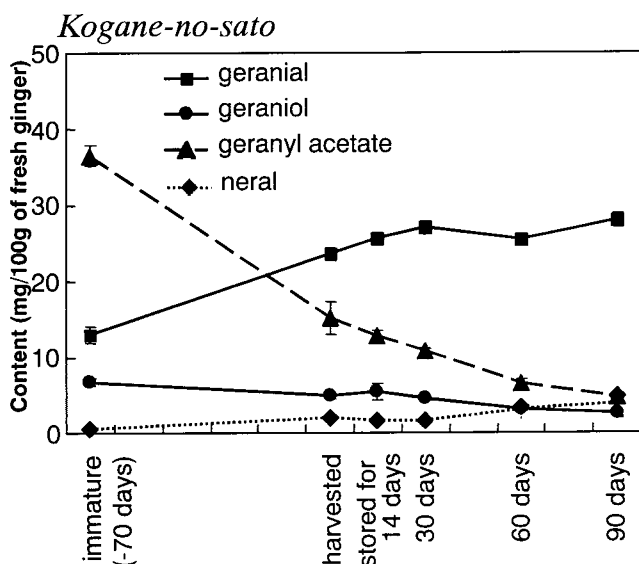


Figure 3. Changes in geraniol-related compounds during maturation and storage of two varieties of ginger (*Tosaichi* and *Kogane-no-sato*). Each value is presented as the mean ± SD ($n = 3$).

in triplicate. A crude enzyme solution was prepared from each acetone powder sample and used for measuring the GeDH activity. The water content changed from 95 to 87 and 89% during the maturing of *Tosaichi* and *Kogane-no-sato*, respectively, but did not change any more during storage. On the other hand, the specific gravity of each ginger sample remained unchanged during maturation and storage.

The changes in the amounts of geranial, neral, geraniol, and geranyl acetate during the maturation and storage of ginger are shown in Figure 3. Almost the same trends were found in both varieties of ginger. The amount of geranial reached the maximum after 30 days of storage, being 48 mg/100 g of fresh ginger for *Tosaichi* and 27 mg/100 g for *Kogane-no-sato*, and then slightly decreased or remained constant with further storage. Geranial was more markedly generated in *Tosaichi* than in *Kogane-no-sato*. On the other hand, the amounts of geranyl acetate and geraniol, respectively, decreased to 16−18 and 42−44% of the initial levels during maturation and storage. Although it has been reported that the percentage of each of these compounds in a ginger aroma extract changed during the maturation and storage of ginger (*20*), we found in this study that the absolute quantity of each compound in ginger also changed. The quantitative changes of geranial, geranyl acetate, and geraniol during the maturation and storage of ginger suggest that geranial was formed not only from geraniol but also from geranyl acetate, although it is difficult to explain how geranyl acetate would be directly converted to geranial by their chemical structures. As a preliminary experiment, we have already found that geranyl acetate decreased during the incubation of a ginger homogenate. It was also observed that geranyl acetate in the acetone powder of ginger was deacetylated after incubation (data are not shown). Consequently, the geranial pathway is presumed to be that geranyl acetate was first deacetylated to geraniol and then that geraniol was successively oxidized to geranial. However, it remains unclear why the geraniol content was more regulated than that of geranial or geranyl acetate. An investigation of the overall pathway from geranyl acetate to geranial is underway, and we are continuing the study of this aspect.

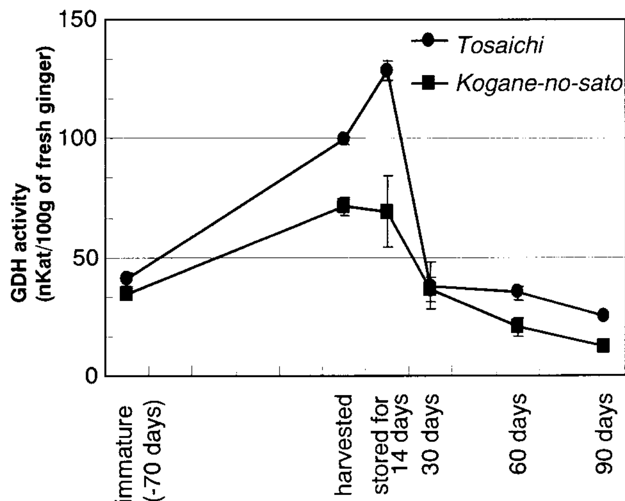


Figure 4. GDH activity of ginger varieties during maturation and storage. Each value is presented as the mean ± SD ($n = 3$).

Figure 4 indicates the change in GeDH activity according to the degree of maturity and storage time of ginger shown per 100 g of fresh ginger weight. The GeDH activity in both varieties of ginger increased with maturity and storage time, changing from 40.1 to 128.3 nkat in *Tosaichi* and from 35.0 to 70.2 nkat in *Kogane-no-sato*, by comparing immature ginger to mature ginger stored for 14 days. However, after further storage, this activity of each sample suddenly decreased to 33.3 nkat (*Tosaichi*) and 32.5 nkat (*Kogane-no-sato*). This change is parallel to that of geranial formation, which increases during maturation and initial storage in Figure 3. In particular, *Tosaichi*, in which geranial increased more, had twice stronger GeDH activity than *Kogane-no-sato*. It is concluded from these results that the GeDH activity that selectively acted on geraniol would have influenced the formation of geranial in ginger. The aroma character of ginger varies throughout the world, and it is considered that the expression and strength of this enzyme activity would lead to the different aroma character of each type of ginger and would be correlated with the flavor quality of ginger. The purification and characterization of GeDH should

be accomplished to enable the rhizomes of ginger to be used more effectively.

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