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# Formation of Flower Fragrance Compounds from Their Precursors by Enzymic Action during Flower Opening

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**Flower fragrance compounds were found to be produced from the precursor solution obtained from flower buds by crude enzyme prepared from the flowers at the opening stage. GC and GC-MS analyses showed the formation of volatile aroma constituents from the precursor solution of *Jasminum polyanthum* F, *Jasminum sambac* Ait, and *Gardenia jasminoides* E, but none in the case of *Osmanthus fragrans* L.**

**The aroma-producing enzyme activity of *G. jasminoides* rapidly increased to reach the maximum at flower opening stage (stage 4) and decreased within 24 h after flower opening (stage 5).**

**Fragrance precursors of *G. jasminoides* were suggested not to be mainly β-glucosides of linalool, eugenol, borneol, and isoeugenol based on the results after β-glucosidase and naringinase treatment of the precursor solution. The activity of hydrolytic enzyme(s) such as glycosidase was found to elevate during flower opening to result in the aroma formation.**

Generally a floral note is released from flowers during anthesis, while flower buds do not smell. Although volatile components of flowers have been intensively analyzed by GC and/or GC-MS, the mechanism of flower fragrance formation has not been well understood. Ackermann *et al.*[1] have suggested that glucosylated monoterpenes were transported from the leaves *via* the phloem to the flowers. Bound forms such as glycosides and phosphates of volatile aroma compounds have been isolated from tea leaves[2,3] and various kinds of fruits,[4–8] and suggested to be direct precursors of volatile aroma components.[9–16] Gunata *et al.*[17] have proposed that in the fruits as well as tea leaves and flowers rutinosides of terpenes were first hydrolyzed by α-rhamnosidase into the glucosides, which were converted into terpenoides by the subsequent enzymic action of β-glucosidase. No direct evidence of enzymic action in flowers was documented until our study on flower fragrance formation was started. Quite recently Loughrin *et al.*[18] have demonstrated that glycosidically bound volatile components of *Nicotiana suaveolens* flowers greatly increased post-anthesis.

Here we propose a hypothetical mechanism of flower fragrance formation as follows. Basically fragrance precursors in flower buds are enzymatically transformed into volatile compounds during flower opening. The enzymes are supposed to be, a) newly induced and expressed; b) activated from the inactive forms, during flower opening. To substantiate the hypothesis we designed an assay protocol as follows. 1) extraction of fragrance precursors from flower buds; 2) preparation of crude enzyme from flowers at the opening stage; 3) reaction of the fragrance precursors with the crude enzyme; 4) ether extraction of the reaction mixture; 5) estimation of the fragrance formation by sensory test and GC and GC-MS analyses of the ether extracts. We called this series of experiments "crude enzyme assay". *Jasminum polyanthum*, *Jasminum sambac*, *Osmanthus fragrans*, and *Gardenia jasminoides* were tested by the crude enzyme assay.

Major aims of this study are (1) to ascertain the formation of the fragrance from the precursor solution by treatment with the crude enzyme and (2) to estimate the amount of the fragrance precursors and the activity of the crude enzymes during flower opening.

## Materials and Methods

*Chemicals.* All the solvents used were distilled before use. β-Glucosidase (from Almond) and naringinase (from *Penicillium decumbens*) were purchased from Sigma. Naringinase contains 300–600 units per g solid as well as β-glucosidase activity up to 150 units per g solid.

*Flower buds and flowers for the crude enzyme assay. Jasminum polyanthum* F. was cultivated at University Farm, Faculty of Agriculture, Shizuoka University. Both the flower buds (29 g fr. wt, 1 to 12 h before flower opening) and flowers (30 g fr. wt, within 24 h after flower opening) were harvested in the morning from March 30 to April 10, 1991. The average flowers (just after opening) with 6–7 mm diameters and 5–6 mm axis weighed *ca.* 60 mg and the buds (1–12 h before opening) with 2–3 × 5–6 mm weighed *ca.* 40 mg.

*Jasminum sambac* Ait was cultivated at Xiaoshan, Zhejiang, China and flower buds (10 kg fr. wt, average weight 370 mg) with the size of 10 × 12 mm were harvested in the morning from August 11 to 15, 1991. The flower buds (200 g fr. wt) were left at 30°C for 10 h to reach a full bloom stage. These flowers had a characteristic jasmine-like floral note, but the flower buds did not.

*Osmanthus fragrans* L.: Flower buds (750 g fr. wt) and flowers (750 g fr. wt) were harvested at Shizuoka, Japan, at 10 to 12 pm on October 6, 1991. Average flower buds 24–48 h before anthesis were 2–3 × 5–6 mm, and flowers at the opening stage were 6–7 × 5–6 mm.

*Gardenia jasminoides* E.: Flowers (500 g fr. wt., average weight 6.0 g, 100 × 110 mm) and flower buds (210 g fr. wt., average weight 5.0 g, 25 × 70 mm), which corresponded to those at stage 5 and stage 3, respectively as described below were harvested in the morning from June 5

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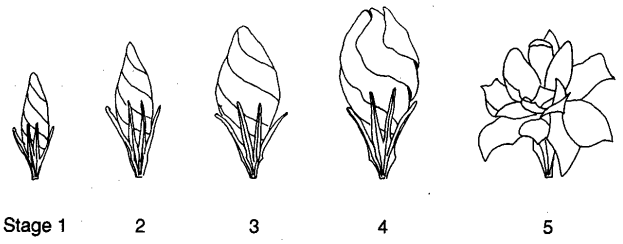


**Fig. 1.** Flowers (Stages 1–5) of *Gardenia Jasminoides* Harvested for the Estimation of Crude Enzyme Activity.

to 20, 1991 at Fujieda, Shizuoka, Japan.

*Gardenia jasminoides E. for evaluation of enzyme activity during flower opening.* Two hundred g each of flower buds and flowers at various flower opening stages of *G. jasminoides* (Fig. 1) were harvested at Fujieda, Shizuoka, Japan in the morning from June 20 to July 10, 1992. Stage 1: immature green buds (average size 13 × 30 mm, average weight 2.9 g); stage 2: mature green buds (15 × 60 mm, 6.1 g, at least 1 to 2 days before opening); stage 3: white buds (17 × 69 mm, 6.4 g, 1 to 12 h before opening); stage 4: flowers (20 × 67 mm, 6.5 g, just after opening); stage 5: flowers (92 × 72 mm, 7.6 g, 12 to 24 h after opening). Flower buds at stages 1, 2, and 3 did not have a gardenia-like floral note, but flowers at stages 4 and 5 had the typical floral note. All the buds and flowers were refrigerated at −20°C in an ice-box immediately after harvest to carry back to our laboratory, and the frozen flowers were treated within 2 h as described below.

*Preparation of crude enzyme for crude enzyme assay.* Flowers were homogenized in 10 × *X* (g fr. wt. expressed as *X*) ml of cold acetone chilled with solid $CO_2$ and filtered. The debris was washed with cold acetone until the flower fragrance almost disappeared. Drying in a dessicator with suction under ice-cooling gave an acetone powder. Yields were as follows: *J. polyanthum* 3 g from 30 g fr. wt; *J. sambac* 50 g from 200 g fr. wt; *O. fragrans* 65 g from 750 g fr. wt; *G. jasminoides* 50 g from 500 g fr. wt. Except in the case of *G. jasminoides*, the acetone powder were directly used as the crude enzyme.

The acetone powder (50 g) of *G. jasminoides* was solubilized in 1000 ml of phosphate buffer (0.1 M, pH 7.0) for 3 h at 4°C. After centrifugation (8000 rpm, 20 min, 4°C) the supernatant (750 ml) was used as a crude enzyme.

*Preparation of crude enzyme for evaluation of activity during flower opening.* Flower buds and flowers of *G. jasminoides* at various stages were treated as described below. From one piece of flower bud or flower at stages 1 to 5, 550, 1100, 1200, 1100, and 1130 mg of acetone powder were obtained, respectively. Protein contents of the acetone powder were not estimated.

*Preparation of precursors solution.* Flower buds just before flower opening were extracted with 80% MeOH three times under ice-cooling. Combined extracts were concentrated *in vacuo* to yield an aqueous residue. As aroma precursors were suggested to be in a bound form such as glycosides or phosphates with low volatility, this was chromatographed on an Amberlite XAD-2 column [column bed volume: $Vt = 12 \times X$ (g dry wt. expressed as *X*) ml]. The column was washed with water (2 × *Vt* ml) and pentane (*Vt* ml) to remove sugars and volatile material that interfere the GC and GC-MS analyses of aroma constituents liberated by the crude enzyme assay. Aroma precursors were eluted with methanol (3 × *Vt* ml) and concentrated. The resulting aq. residue was diluted with water and used for the crude enzyme assay.

*Purification of precursor solution from G. jasminoides.* Flower buds (2.7 kg) at stage 3 of *G. jasminoides* were homogenized in ice-cooled 80% aq. MeOH. After filtration the extract was concentrated *in vacuo* and the resultant aq. solution was diluted with water. A portion (equivalent to 690 g fr. wt.) of the solution (690 ml) was chromatographed on an Amberlite XAD-2 column (45 × 188 mm, $Vt = 300$ ml, equilibrated with water). The column was developed successively with water (700 ml), pentane (300 ml), EtOAc (400 ml), and MeOH (900 ml). Evaporation of the combined EtOAc and MeOH fractions afforded 10.5 g of crude materials, which was subjected to chromatographed on a Sephadex LH-20 column ($Vt = 1100$ ml, 40 mm × 875 mm; 50% aq. MeOH). Fraction D-3 (*Ve*: elution volume/$Vt = 0.7$–$0.9$) was found to contain aroma precursors based on the crude enzyme assay and treatment with β-glucosidase and naringinase.

*Crude enzyme assay.* Group (A): to 10 ml of a precursor solution (equivalent to 20 g of flower buds) was added with crude enzyme solution (3.75 ml, equivalent to 250 mg of flowers) or acetone powder (equivalent to 200 mg of flowers), (B) precursor solution + inactivated crude enzyme (heated for 1 min by a microwave oven), (C) precursor solution [precursor solution + 0.1 M citrate buffer (pH 5.0)], (D) crude enzyme + 0.1 M citrate buffer (pH 5.0), (E) inactivated crude enzyme + citrate buffer, (F) precursor solution + β-glucosidase (1 mg) and (G) precursor solution + nariginase (1 mg). Each reaction mixture was filled up to 20 ml with 0.1 M citrate buffer (pH 5.0) and was incubated at 30°C in the presence of sodium azide (500 μg/ml). Duplicate sets of groups were assayed as follows: one was for sensory test and the other was for GC and GC-MS analyses. After 72 h the reaction mixture was filtered and 0.5 ml of ether solution of ethyl octanoate (20 μg/ml) was added to the reaction mixture as an internal standard. Aroma liberated from the precursor solution during incubation were extracted twice with ether. Combined extract was dried over anhydrous $Na_2SO_4$ and concentrated to a minimum volume by a $N_2$ stream. Concentrated material was directly analyzed by GC and GC-MS.

*Evaluation of enzyme activity during flower opening.* In this assay were used acetone powder equivalent to 0.5 piece of *G. jasmimoides* flower buds or flowers. The precursor solution (equivalent to 10 g fr. wt./15 ml of 0.1 M citrate buffer, pH 5.0) was prepared from the buds at stage 3. According to the method above mentioned the variation of crude enzyme activity was estimated.

*Sensory test.* A strip (5 × 50 mm) of filter paper was dipped into an ether extract and tested. At least three volunteers judged whether samples gave a floral note or not.

*GC and GC-MS analyses.* A Hitachi model G-3000 gas chromatograph with a FID detector, equipped with a capillary column of TC-WAX or PEG-20M (GL Science Co. Ltd., Japan, 0.25 mm i.d. × 30 or 25 m) and a recorder (JASCO Model 807-IT, Japan) was used. Column temperature was held at 60°C for 10 min, increased to 200°C at a program rate of 3°C/min, and then held for 20 min at 200°C. Injector and detector temperature were 250°C, respectively. $N_2$ at 1 ml/min was used as a carrier gas. An ethereal sample solution (0.8 μl) was injected through a splitter (1 : 90). For GC-MS, a JMS DX-302 GC-MS spectrometer (JEOL, Japan) with the same capillary column and a data acquisition system DA-3000 was used. Analyzing parameters were as follows: ionizing voltage: 70 eV; accelerating voltage: 3 kV; mass scan rate: 1 sec for *m/z* 40–500. Results of analyses were verified by comparison of relative retention time and mass spectral data with those of authentic specimens and a relative peak intensity to that of an internal standard (ethyl octanoate).

## Results and Discussion

### *Sensory test after crude enzyme assay*

As shown in Table I crude enzyme treated samples (A) liberated floral note except the *O. fragrans*, but samples (B)–(E) did not smell in the sensory test in all the flowers. β-D-Glucosides and α-L-rhamnopyranosyl-β-D-glucosides of some terpenoides and benzyl alcohol have been reported to be candidates for aroma precursors in tea leaves and fruits.[2-16] To examine whether the flower fragrance were produced by the action of β-glucosidase or/and naringinase, each precursor solution was treated with these enzymes. In the sensory test naringinase-treated samples (G) had more fragrant notes than β-glucosidase-treated ones (F) as shown in Table I. In *G. jasminoides*, *J. sambac*, and *J. polyanthum* samples (A) had the highest floral note among the tested samples (A)–(G). Thus the floral note from the flowers were suggested to be produced by some other enzymatic action in addition to β-glucosidase.

### *GC and GC-MS analyses after crude enzyme assay*

Tables II–IV show the relative peak intensities of

**Table I.** Results of the Sensory Test in the Crude Enzyme Assay of the Precursor Extracts from *Jasminum polyanthum*, *Jasminum sambac*, *Gardenia jasminoides*, and *Osmanthus fragrans*

| | Reaction mixtures | *J. poly* | *J. sam* | *G. jas* | *O. frag* |
|---|---|---|---|---|---|
| (A) | acetone powder + precursor extract | ◎ | ◎ | ◎ | × |
| (B) | heated acetone powder + precursor extract | × | × | × | × |
| (C) | precursor extract + buffer* | × | × | × | × |
| (D) | acetone powder + buffer | × | × | × | × |
| (E) | heated acetone powder + buffer | × | × | × | × |
| (F) | (C) + β-glucosidase | ○ | ○ | ○ | ○ |
| (G) | (C) + naringinase | ◎ | ◎ | ◎ | ○ |

◎, floral note; ○, weaker than ◎; ×, grassy or no smell.
* 50 mM citrate buffer (pH 5.0).

**Table II.** Gas Chromatographic Analytical Data of Aroma Compounds Liberated from the Precursor Solution Prepared from *Jasminum polyanthum*, by the Action of Its Crude Enzyme Preparation, β-Glucosidase and Naringinase

| Peak No. | Relative $t_R$ | Compounds | (A)[a] | (C) | (D) | (F) | (G) |
|---|---|---|---|---|---|---|---|
| 1 | 0.20 | *[b] | 1.9[c] | — | — | 0.1 | 0.9 |
| 2 | 0.25 | * | 6.7 | — | 0.4 | — | — |
| 3 | 0.31 | * | 1.0 | — | — | — | — |
| 4 | 0.47 | * | 2.5 | — | 0.6 | — | — |
| 5 | 0.55 | * | — | — | — | — | — |
| 6 | 0.66 | * | 0.1 | — | — | — | — |
| 7 | 0.76 | * | 0.1 | — | — | 0.1 | 0.9 |
| 8 | 0.84 | * | 2.6 | — | 1.9 | — | — |
| 9 | 0.89 | (*Z*)-3-Hexenol | 0.2 | — | — | 0.1 | 0.6 |
| 10 | 1.00 | Ethyl octanoate | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 11 | 1.24 | Linalool | 0.1 | — | — | — | 0.7 |
| 12 | 1.82 | Benzyl alcohol | 2.5 | — | 0.4 | 1.4 | 8.9 |
| 13 | 1.87 | 2-Phenylethanol | 4.7 | 0.5 | 3.3 | 3.0 | 50.0 |
| 14 | 1.95 | 2-Methoxy-*p*-cresol | 1.0 | — | 0.1 | 5.0 | 6.1 |
| 15 | 2.15 | *p*-Cresol | 3.1 | — | 0.1 | 7.7 | 12.6 |
| 16 | 2.26 | Eugenol | 1.4 | — | 0.1 | 2.6 | 3.7 |
| 17 | 2.45 | * | 0.8 | — | 0.1 | 1.4 | 1.7 |
| 18 | 2.50 | (*E*)-Isoeugenol | 0.9 | — | 0.1 | 2.3 | 3.4 |
| 19 | 2.59 | * | 1.1 | — | 0.2 | — | 0.6 |

[a] Refer to Table I.
[b] Unidentified peak.
[c] Data are calibrated relative intensities so that the relative peak intensity due to an internal standard (ethyl octanoate) is 1.0.

**Table III.** Gas Chromatographic Analytical Data of Aroma Compounds Liberated from the Precursor Solution Prepared from *Gardenia jasminoides*, by the Action of Its Crude Enzyme Preparation, β-Glucosidase and Naringinase

| Peak No. | Relative $t_R$ | Compounds | (A)[a] | (B) | (C) | (D) | (E) | (F) | (G) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.70 | *[b] | 1.4[c] | — | — | — | — | — | NT |
| 2 | 0.85 | * | 0.2 | — | — | — | — | 0.1 | NT |
| 3 | 0.92 | (*Z*)-3-Hexenol | 1.3 | — | — | 0.1 | — | 0.7 | NT |
| 4 | 1.00 | Ethyl octanoate | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | NT |
| 5 | 1.05 | * | 0.4 | — | — | — | — | — | NT |
| 6 | 1.22 | Linalool | 0.3 | — | — | — | — | — | NT |
| 7 | 1.29 | * | 2.8 | 0.1 | 1.0 | 0.1 | 0.1 | 0.1 | NT |
| 8 | 1.44 | * | 1.7 | 0.5 | 0.3 | 0.2 | 0.2 | 2.1 | NT |
| 9 | 1.49 | Borneol | 0.5 | — | — | — | — | — | NT |
| 10 | 1.71 | * | 0.4 | 0.1 | 0.1 | — | — | 1.5 | NT |
| 11 | 1.76 | Benzyl alcohol | 0.4 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | NT |
| 12 | 1.80 | 2-Phenylethanol | 2.1 | 0.1 | 0.1 | — | — | 0.4 | NT |
| 13 | 2.16 | Eugenol | 0.1 | — | — | — | — | — | NT |

[a] Refer to Table I.
[b] Unidentified peak.
[c] Data are calibrated relative intensities so that the relative peak intensity due to an internal standard (ethyl octanoate) is 1.0.

individual peaks to the internal standard in each GC analytical data of aroma compounds liberated from the precursor solutions by the action of the crude enzyme preparation from flowers and buds, β-glucosidase and naringinase. Thus the comparison of the data makes possible to estimate the quantities of fragrant compounds which were formed enzymatically or non-enzymatically from the precursor solutions.

*Jasminum polyanthum*

As shown in Table II the crude enzyme prepared from *J. polyanthum* flowers (A) transformed the precursors to (*Z*)-3-hexenol, linalool and other unidentified volatiles, which were not detected in the control groups; namely, treatments (C) and (D). Besides them following compounds significantly increased in (A) by several times (expressed as × a) compared with those in the groups (C) or (D): benzyl alcohol (×6.3), 2-phenylethanol (×1.4), 2-methoxy-*p*-cresol (×10), *p*-cresol (×31), eugenol (×14), (*E*)-isoeugenol (×9), and at least five unidentified compounds (×1.3–17). β-Glucosidase treatment (F) produced (*Z*)-3-hexenol, benzyl alcohol, 2-methoxy-*p*-cresol, eugenol, and (*E*)-isoeugenol. 2-Phenylethanol and *p*-cresol increased by 6 and 77 times, respectively, compared with those in the precursor solution (C) or (D). Naringinase (G), much more effectively than β-glucosidase, transformed the bound form precursors into linalool and an unidentified compound (relative $t_R$ 2.59) besides the compounds produced by β-glucoside. These data supported the results of the sensory test in order (A) > (G) > (F) ≫ (B, C, D, E). Thus the precursors of these aroma constituents are suggested to be mainly their rhamnosides, rutinosides, and/or glucosides.

Although steam-distillates obtained by SDE (simultaneous distillation and extraction) (data not shown) of this flower contained benzyl acetate and farnesol as major components, crude enzyme and hydrolytic enzyme did not produce these compounds from the precursor solution. The putative biosynthetic precursors of these compounds are the corresponding alcohol and acid, and the phosphate(s), respectively. Thus the procedures used in this experiment are not appropriate for the formation of these aromas.

**Table IV.** Gas Chromatographic Analytical Data of Aroma Compounds Liberated from the Precursor-rich Fraction D-3 Prepared from the Precursor Solution of *Gardenia jasminoides*, by the Action of Its Crude Enzyme Preparation, β-Glucosidase and Naringinase

| Peak No. | Relative $t_R$ | Compounds | (A)[a] | (C) | (F) | (G) |
|---|---|---|---|---|---|---|
| 1 | 0.72 | *[b] | 0.1[c] | — | — | 0.1 |
| 2 | 0.84 | * | 0.4 | — | 0.2 | 0.2 |
| 3 | 1.00 | Ethyl octanoate | 1.0 | 1.0 | 1.0 | 1.0 |
| 4 | 1.41 | Linalool | 1.1 | — | 0.7 | 5.2 |
| 5 | 1.76 | * | 0.2 | — | 0.3 | 2.1 |
| 6 | 1.84 | Borneol | 2.0 | 0.1 | 0.1 | 3.8 |
| 7 | 2.38 | Benzyl alcohol | 1.0 | 0.2 | 0.4 | 0.4 |
| 8 | 2.48 | 2-Phenylethanol | 3.3 | — | 0.6 | 7.4 |
| 9 | 3.03 | Eugenol | 0.4 | 0.1 | 0.1 | 1.0 |
| 10 | 3.17 | * | — | 0.1 | 0.2 | 1.1 |
| 11 | 3.14 | * | — | — | 0.3 | 0.7 |
| 12 | 3.52 | * | — | — | — | 0.3 |

[a] Refer to Table I.
[b] Unidentified peak.
[c] Data are calibrated relative intensities so that the relative peak intensity due to an internal standard (ethyl octanoate) is 1.0.

*Gardenia jasminoides*

Table III shows the results on *G. jasminoides*. Crude enzyme treatment of the precursor solution (A) gave as main volatiles, linalool, borneol, eugenol, and two unidentified compounds, which were not detected in groups (C) and (D). Besides them, benzyl alcohol + BHT (×2), 2-phenylethanol (×21) and three unidentified compounds (peaks 7, 8, and 10; ×2.8, 5.7, and 4.0, respectively) significantly increased.

Partial purification of the precursor solution by chromatography on an Amberlite XAD-2 and Sephadex LH-20 columns yielded a precursor-rich fraction (D-3). As shown in Table IV linalool and 2-phenylethanol were newly detected in group (A). Besides these compounds, borneol (×20) and eugenol (×4) were unambiguously increased by the crude enzyme treatment of this fraction (D-3). Similar results were obtained by naringinase treatment (G), while β-glucosidase (F) produced only linalool and 2-phenylethanol but did not borneol and eugenol. Amounts of borneol and eugenol liberated by naringinase increased by 35 and 14 times, respectively. When *p*-nitrophenyl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside (Rha-Glc-*p*NP) was treated with the crude enzyme, it was not hydrolyzed (unpublished data). Naringinase liberated *p*NP from Rha-Glc-*p*NP. These observation suggest that the crude enzyme contains some hydrolytic enzyme besides β-glucosidase, rather than α-rhamnosidase. Naringinase is suggested to show α-rhamnosidase, β-glucosidase, and other hydrolytic enzyme activity, as previously reported by Gunata *et al.*[17] Therefore, the precursors of linalool are suggested to be its glycoside as well as the glucosides. Borneol and eugenol seemed to be present as their glycosides rather than their glucoside.

Although linalool, a characteristic and abundant fragrant constituent, was produced by the enzymic reaction, esters like tiglic acid esters, jasmine lactone, methyl benzoate, and (*Z*)-3-hexenyl benzoate *etc.* characteristic of *G. jasminoides* were not detected.

**Table V.** Gas Chromatographic Analytical Data of Aroma Compounds Liberated from the Precursor Solution Prepared from *Jasminum sambac*, by the Action of Its Crude Enzyme Preparation, β-Glucosidase, and Naringinase

| Peak No. | Relative $t_R$ | Compound | (A)[a] | (C) | (D) | (F) | (G) |
|---|---|---|---|---|---|---|---|
| 1 | 0.25 | *[b] | 8.8[c] | — | — | 1.6 | 2.1 |
| 2 | 0.44 | * | 3.2 | 1.1 | — | 2.0 | 2.0 |
| 3 | 0.46 | * | 5.0 | — | — | — | — |
| 4 | 0.86 | 4-OH-4-Me-2-pentanone | 24.8 | 0.1 | 0.4 | 0.1 | — |
| 5 | 0.89 | * | 1.9 | — | — | 0.3 | — |
| 6 | 0.91 | (*Z*)-3-Hexenol | 2.7 | — | — | 5.2 | 7.7 |
| 7 | 1.00 | Ethyl octanoate | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 8 | 1.09 | Acetic acid | 59.4 | 0.3 | 0.7 | 0.7 | 0.6 |
| 9 | 1.27 | Linalool | 1.4 | 0.3 | — | 0.5 | 3.7 |
| 10 | 1.53 | * | 1.3 | 0.3 | — | 0.3 | — |
| 11 | 1.54 | * | 1.2 | 0.2 | — | 0.2 | — |
| 12 | 1.69 | * | 49.1 | 6.0 | — | 1.1 | 4.7 |
| 13 | 1.80 | * | 1.4 | — | — | 0.2 | — |
| 14 | 1.90 | Benzyl alcohol | 35.3 | 0.2 | 0.7 | 2.6 | 21.8 |
| 15 | 1.95 | 2-Phenylethanol | 6.4 | 1.2 | — | 5.7 | 41.7 |
| 16 | 1.99 | * | 0.8 | — | — | — | — |
| 17 | 2.02 | * | 0.8 | 0.9 | — | 0.7 | — |
| 18 | 2.04 | * | 4.3 | 2.2 | — | 1.5 | 1.1 |
| 19 | 2.06 | * | 20.1 | 2.6 | — | 2.8 | 1.4 |
| 20 | 2.07 | * | 0.8 | — | — | — | — |
| 21 | 2.17 | * | 16.6 | 1.2 | — | 0.8 | 0.5 |
| 22 | 2.23 | * | 1.5 | 1.0 | — | 0.4 | — |
| 23 | 2.32 | * | 1.0 | 0.7 | — | 0.3 | — |
| 24 | 2.46 | Methyl anthranilate | 10.7 | 6.4 | 2.8 | — | — |
| 25 | 2.53 | * | 5.0 | 1.8 | — | 1.8 | 1.5 |
| 26 | 2.57 | * | 1.4 | 0.7 | — | 1.0 | 0.8 |
| 27 | 2.59 | * | 18.6 | 9.7 | — | 9.8 | 13.0 |
| 28 | 2.62 | * | 0.9 | — | — | — | — |
| 29 | 2.70 | * | 2.9 | 0.6 | — | 0.6 | 0.8 |
| 30 | 2.79 | * | 2.1 | 0.4 | — | — | — |
| 31 | 2.81 | * | 1.7 | — | — | — | — |
| 32 | 3.03 | * | 1.8 | 1.8 | — | 1.0 | 0.6 |

[a] Refer to Table I.
[b] Unidentified peak.
[c] Data are calibrated relative intensities so that the relative peak intensity due to an internal standard (ethyl octanoate) is 1.0.

**Table VI.** Gas Chromatographic Analytical Data of Aroma Compounds Liberated from the Precursor Solution Prepared from *Osmanthus fragrance*, by the Action of Its Crude Enzyme Preparation, β-Glucosidase, and Naringinase

| Peak No. | Relative $t_R$ | Compounds | (A)[a] | (C) | (D) | (F) | (G) |
|---|---|---|---|---|---|---|---|
| 1 | 0.82 | (*Z*)-3-Hexenol | 0.2[b] | — | — | 0.5 | 0.8 |
| 2 | 1.00 | Ethyl octanoate | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 3 | 2.41 | BHT | 8.6 | 3.3 | 5.5 | 2.3 | 8.0 |
| 4 | 2.51 | *[c] | 0.4 | 0.6 | 0.5 | 0.3 | 3.8 |
| 5 | 2.57 | * | — | — | — | — | 1.0 |
| 6 | 3.55 | * | 0.7 | 1.7 | — | 2.1 | 5.5 |

[a] Refer to Table I.
[b] Data are calibrated relative intensities so that the relative peak intensity due to an internal standard (ethyl octanoate) is 1.0.
[c] Unidentified peak.

*Jasminum sambac*

Table V shows the results for *J. sambac*. Besides five newly produced unidentified compounds, 4-hydroxy-4-

**Table VII.** Qualitative and Quantitative Changes[a] in Volatile Compounds Liberated by the Crude Enzyme Treatment during the Development of *Gardenia jasminoides* Flowers

| Peak No. | Relative $t_R$ | Compound | P[b] | Stage 1 | | Stage 2 | | Stage 3 | | Stage 4 | | Stage 5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | (C)[c] | (D) | (A) | (D) | (A) | (D) | (A) | (D) | (A) | (D) | (A) |
| 1 | 0.25 | *[d] | 1.3 | — | 0.6 | — | 0.4 | — | 1.2 | — | — | — | — |
| 2 | 0.37 | * | 0.3 | 3.1 | 4.8 | 2.3 | 2.5 | 3.7 | 3.5 | — | 5.3 | — | — |
| 3 | 0.80 | * | — | — | — | — | — | — | 0.2 | — | 0.5 | — | 0.1 |
| 4 | 0.82 | * | — | 0.6 | 0.6 | — | 0.2 | — | 0.2 | — | 0.4 | 0.5 | 0.3 |
| 5 | 0.90 | (*Z*)-3-Hexenol | — | — | 0.7 | — | 0.7 | 0.4 | 1.6 | 0.9 | 5.3 | 1.0 | 1.3 |
| 6 | 1.00 | Ethyl octanoate | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 7 | 1.07 | * | — | — | — | — | — | — | 0.1 | — | 2.3 | 0.1 | — |
| 8 | 1.25 | Linalool | — | — | 0.2 | 0.1 | 0.6 | 0.3 | 1.0 | 0.6 | 3.1 | 0.3 | 1.7 |
| 9 | 1.35 | * | 0.3 | — | — | 0.1 | — | — | 0.2 | — | 0.2 | 0.1 | 0.3 |
| 10 | 1.50 | * | — | — | — | 0.1 | 0.1 | 0.6 | 0.9 | 0.9 | 1.6 | 0.6 | 0.7 |
| 11 | 1.50 | Borneol | 0.4 | 0.2 | 0.2 | 0.1 | 0.4 | — | 0.9 | — | 1.7 | 0.2 | 1.2 |
| 12 | 1.70 | 1-Phenylethanol | 0.2 | 0.2 | — | 0.1 | 0.1 | — | 0.3 | — | 0.5 | 0.2 | 0.5 |
| 13 | 1.80 | Benzyl alcohol | — | — | — | — | — | 0.2 | 0.2 | 0.4 | 0.4 | 0.2 | 0.2 |
| 14 | 1.90 | 2-Phenylethanol | 1.9 | 0.4 | 1.3 | 0.3 | 1.9 | 0.8 | 3.7 | 0.4 | 6.7 | 0.4 | 5.1 |
| 15 | 2.30 | Eugenol | — | 0.1 | — | 0.1 | 0.2 | — | 0.3 | 0.4 | 0.5 | 0.1 | 0.6 |
| 16 | 2.35 | * | — | — | — | — | 0.1 | — | 0.2 | — | 5.5 | 0.1 | 0.6 |
| 17 | 2.53 | * | — | — | — | — | 0.1 | — | 0.2 | — | 0.4 | — | 0.4 |
| 18 | 2.80 | * | — | — | — | — | 0.5 | — | 0.3 | 0.1 | — | — | — |

[a] Data are calibrated relative intensities so that the relative peak intensity due to an internal standard (ethyl octanoate) is 1.0.
[b] Precursor extracts (Stage 3) + buffer.
[c] Refer to Table I.
[d] Unidentified peak.

methyl-2-pentanone (derived from acetone), (*Z*)-3-hexenol, linalool, and benzyl alcohol were clearly increased by the crude enzyme treatment (A). Naringinase treatment (G) yielded higher amounts of linalool and 2-phenylethanol than β-glucosidase treatment (F), but higher level of benzyl alcohol was detected in (F), suggesting that the precursors of linalool and 2-phenylethanol are their rutinosides or rhamnosides rather than their glucosides. Benzyl alcohol and (*Z*)-3-hexenol are suggested to be present as their glucosides.

As with the other flowers, benzyl acetate, one of the characteristic and dominant fragrances of *J. sambac*, could not be detected.

*Osmanthus fragrans*

Unlike the three kinds of flowers mentioned above, as shown in Table VI, crude enzyme prepared from *O. fragrans* produced neither fragrant components nor major constituents in the SDE extract, such as linalool, linalool oxides, α- and β-ionones, or dihydro-β-ionone from the precursor solution. The presence of the glycosides of (*Z*)-3-hexenol, 2-phenylethanol and some compounds in the precursor solution was suggested based on the GC traces after β-glucosidase (F) and naringinase (G) treatment.

*Changes in activity of crude enzymes during flower opening*

We have demonstrated above that there are considerable amounts of the glycosidic precursors in the mature flower buds, and that the crude enzyme activity of the opening flowers is high enough to hydrolyze the precursors to yield fragrant compounds. The next subject of this study is to clarify whether the activity of crude enzyme changes during the growth and development of flower buds and their opening.

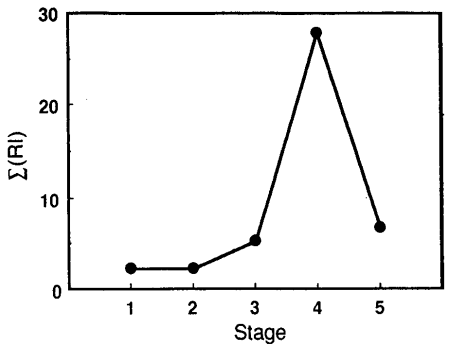


**Fig. 2.** Changes of Volatile Components of *Gardenia Jasminoides* Liberated by Treatment with the Crude Enzymes Prepared from the Various Stages of the Flower Buds and Flowers during the Opening.

Vertival axis: ∑RI indicates ∑Peak increments relative to the internal standard (ethyl octanoate) = ∑Peak intensity relative to the internal standard (ethyl octanoate) [(A)–(C)–(D) in Table II].

Flower buds and flowers of *G. jasminoides* were harvested at various growth stages (stages 1–5, Fig. 1) and their crude enzymes were prepared. The precursor solution from flower buds (white buds without floral note) at stage 3 (1–12 h before anthesis) was treated with the crude enzymes at each stage. The reaction mixture was extracted with ether to give a sample for GC analyses. A good floral note was detected in the ether extracts obtained after treatment with enzymes at stages 4 and 5. Flowers at stages 4 and 5 also had a floral note.

Table VII shows detected compounds and their relative amounts produced by the action of the crude enzyme at each stage. The dominant compounds detected were (*Z*)-3-hexenol, linalool, borneol, 1-phenylethanol and 2-phenylethanol and unidentified compounds **2**, **3**, **4**, **7**, **16**, **17**, and **18**. The contents of these compounds were almost negligible at stages 1 and 2 and gradually increased (stage

3) during the development of flower buds. Just before flower opening (between stages 3 and 4) they dramatically increased to reach their maximum levels (stage 4) and greatly decreased within 24 h (stage 5) after flower opening. Summing up their relative amounts at each stage must represent the crude enzyme activity at each stage as shown in Fig. 2. Considering that the precursors are glycosidically bound forms, hydrolytic enzymes were activated or induced and expressed their activity during flower opening to produce these fragrant compounds. Clarifying these phenomena on the hydrolytic enzymes during flower opening will become a clue to reveal the still unclear mechanism of anthesis. For this purpose we are now isolating the direct precursors of the volatile compounds and glycosidase-like enzymes using the crude enzyme assay.

The enzyme activity at stage 5 was found to be low, although the *G. jasminoides* flower maintains its floral note for 2 to 4 days. This suggested that the accumulated precursors of volatile compounds, as suggested by Loughrin *et al.*,[18] might be gradually transformed into the volatiles by the enzymic action after flower opening. It is also essential to clarify the changes of precursors' contents in flower buds and flowers of *G. jasminoides* during the growth, development, and anthesis.

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## References

1) I. E. Ackermann, D. V. Banthrope, W. D. Fordham, J. P. Kinder, and I. Poots, *J. Plant Physiol.*, **134**, 567–571 (1989).
2) M. Yano, K. Okada, K. Kubota, and A. Kobayashi, *Agric. Biol. Chem.*, **54**, 1023–1028 (1990).
3) M. Yano, Y. Joki, H. Mutoh, K. Kubota, and A. Kobayashi, *Agric. Biol. Chem.*, **55**, 1205–1206 (1991).
4) P. J. Williams, C. R. Strauss, B. Wilson, and R. A. Massy-Westropp, *Phytochemistry*, **21**, 2013–2020 (1982).
5) W. Schwab and P. Schreier, *Phytochemistry*, **29**, 161–164 (1990).
6) S. Voirin, R. Baumes, S. Bitteur, Z. Gunata, and C. Bayonove, *J. Agric. Food Chem.*, **38**, 1373–1378 (1990).
7) A. Pabst, D. Barron, E. Semon, and P. Schreier, *Phytochemistry*, **31**, 3105–3017 (1992).
8) N. D. Tommasi, R. Aquino, F. D. Simone, and C. Pizza, *J. Natural Products*, **55**, 1025–1032 (1992).
9) T. Takeo, *Phytochemistry*, **20**, 2145–2147 (1981).
10) P. J. Williams, C. R. Strauss, B. Wilson, and R. A. Massy-Westropp, *J. Agric. Food Chem.*, **30**, 1219–1223 (1982).
11) W. Schwab, C. Mahr, and P. Schreier, *J. Agric. Food Chem.*, **37**, 1009–1012 (1989).
12) R. G. Buttery, G. Takeoka, R. Teranishi, and L. C. Ling, *J. Agric. Food Chem.*, **38**, 2050–2053 (1990).
13) P. Wu, M. C. Kuo, T. G. Hartman, R. T. Rosen, and C. T. Ho, *J. Agric. Food Chem.*, **39**, 170–172 (1991).
14) A. Pabst, D. Barron, P. Etievant, and P. Schreier, *J. Agric. Food Chem.*, **39**, 173–175 (1991).
15) S. K. Park, J. C. Morrison, D. O. Adams, and A. C. Noble, *J. Agric. Food Chem.*, **39**, 514–518 (1991).
16) G. Krammer, P. Winterhalten, M. Schwab, and P. Schreier, *J. Agric. Food Chem.*, **39**, 778–781 (1991).
17) Z. Gunata, S. Bitteur, R. Baumes, J. M. Brillouet, C. Tapiero, C. Bayonove, and R. Cordonnier, Eur. Patent, 0 332 281 A1 (March 8, 1989).
18) J. H. Loughrin, T. R. Hamilton-Kemp, H. R. Burton, R. A. Andersen, and D. F. Hildebrand, *Phytochemistry*, **31**, 1537–1540 (1992).