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Characterization of Coumarin-Specific Prenyltransferase Activities in *Citrus limon* Peel

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Coumarins, a large group of polyphenols, play important roles in the defense mechanisms of plants, and they also exhibit various biological activities beneficial to human health, often enhanced by prenylation. Despite the high abundance of prenylated coumarins in citrus fruits, there has been no report on coumarin-specific prenyltransferase activity in citrus. In this study, we detected both *O*- and *C*-prenyltransferase activities of coumarin substrates in a microsome fraction prepared from lemon (*Citrus limon*) peel, where large amounts of prenylated coumarins accumulate. Bergaptol was the most preferred substrate out of various coumarin derivatives tested, and geranyl diphosphate (GPP) was accepted exclusively as prenyl donor substrate. Further enzymatic characterization of bergaptol 5-*O*-geranyltransferase activity revealed its unique properties: apparent K_m values for GPP (9 μM) and bergaptol (140 μM) and a broad divalent cation requirement. These findings provide information towards the discovery of a yet unidentified coumarin-specific prenyltransferase gene.

Key words: aromatic substrate prenyltransferase; coumarin; lemon fruit; bergamottin

Coumarins (α-benzopyrones) form a large group of plant polyphenols. Thus far about 1,500 coumarin derivatives have been isolated from plants.[1)] They occur ubiquitously in the plant kingdom, and coumarin derivatives show a chemotaxonomical tendency to accumulate in large amounts in Rutaceae and Apiaceae.[2)] They exhibit a variety of biological functions, including chemoprevention against pathogens,[3)] herbivores,[4)] and abiotic stresses,[5)] suggesting that physiological roles of coumarins for plants in the adaption to environmental stresses. Some coumarin derivatives are also known to act beneficially on human health due to their therapeutic effects, *e.g.*, inhibitory activities against various tumor cells[6,7)] and mycobacteria,[8)] which have been extensively studied in the medical and pharmaceutical fields for the treatment of human disorders. On the other hand, coumarins are recognized as undesirable compounds in citrus juice and fragrances containing citrus essential oils. This is mostly because they inhibit CYP3A4, an enzyme that plays an important role in metabolizing xenobiotics, including orally administered medicines,[9)] and because they cause skin diseases *via* photosensitivity under UV-A.[10,11)] Thus, despite several desirable activities of coumarins in human health, reducing their contents in citrus fruits is of interest to many researchers in the food sciences and cosmetics companies, whereas from the viewpoint of basic science, many genes and enzymes in the coumarin biosynthetic pathway have not been elucidated.

In plant cells, almost all coumarin molecules undergo modifications, glycosylation, prenylation, hydroxylation, and methylation. Among these, prenylation contributes greatly to the chemical diversification of coumarin derivatives through a variety of prenylation positions, prenyl chain lengths, and further modifications of the prenyl chain. Prenylation represents the key reaction step in the formation of the basic skeleton of furanocoumarins, a subfamily of coumarin derivatives. Precursor feeding experiments have indicated that the prenylation of umbelliferone at 6- or 8-position is the determining reaction step in the formation of linear furanocoumarins and angular furanocoumarins respectively.[12)] Moreover, prenylation contributes to enhancement of the biological activities of coumarins. One example of such enhancement is the furanocoumarin xanthotoxol. When xanthotoxol is dimethylallylated to imperatorin, inhibitory activity against cytochrome P450 of the *Manduca sexta* midgut increases by more than 100-fold.[4)] When it is geranylated to 8-geranyloxypsoralen, additional antimicrobial activity against various mycobacteria species is seen.[8)] In sum, prenyltransferases have a special importance in coumarin biosynthesis.

Plant-derived prenyltransferases for aromatic metabolites have been studied for more than four decades. In previous studies by Yamamoto *et al.* on prenyltransfer-

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Abbreviations: DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate

ases for flavonoids, dimethylallyltransferase activities for naringenin and leachianone G were characterized biochemically in a leguminous medicinal plant, *Sophora flavescens*, and the biosynthesis pathway of a major prenylated flavanone, sophoraflavanone G, was elucidated in plant cell culture.[13,14] In the soybean (*Glycine max*), dimethylallyltransferase activities play crucial roles in the biosynthesis of various phytoalexins, *e.g.*, glyceollins, in which the prenylation of a flavonoid skeleton is involved.[15] As for the prenyltransferases of coumarins, umbelliferone 6-dimethylallyltransferase activity and umbelliferone 7-*O*-dimethylallyltransferase activity have been reported in *Ruta graveolens*,[16,17] and *Ammi majus*[18] respectively, but there has been no report on coumarin-prenyltransferase activities in citrus species, regardless of their importance in basic and applied science. In addition, novel geranyltransferases recognizing aromatic compounds as substrates have not been characterized thus far, except for that of the shikonin biosynthetic enzyme LePGT1 in *Lithospermum erythrorhizon*.[19] Moreover, as for *O*-prenyltransferase, there is only one report on the aforementioned umbelliferone 7-*O*-dimethylallyltransferase activity.[18] In the present study, we characterized biochemically the coumarin-prenyltransferase activity in the flavedo, the outer part of the lemon (*Citrus limon*) fruit peel, where large amounts of prenylated coumarins accumulate.

Materials and Methods

Plant materials and chemicals. Lemon (*C. limon* cv. Lisbon) fruit was purchased at a local market (Kyoto, Japan) and used in the preparation of crude enzymes. Bergaptol, xanthotoxol, bergamottin, and 5-geranyloxy-7-methoxycoumarin were from Extrasynthese (Lyon, France). 5-Hydroxy-7-methoxycoumarin, 5-methoxy-7-hydroxycoumarin and 5,7-dihydroxycoumarin were from Herboreal (Edinburgh, UK). Umbelliferone was from Wako Pure Chemical Industries (Osaka, Japan), and 8-geranyloxypsoralen (xanthotoxol geranyl ether) was from Indofine (Hillsborough, New Jersey). 8-Geranylumbelliferone was synthesized as described in a previous report.[20] Dimethylallyl diphosphate (DMAPP) was synthesized chemically as previously described.[21] Geranyl diphosphate (GPP) was provided by Dr. T. Kuzuyama (The University of Tokyo) and Dr. T. Kawasaki (Kobe University). The other chemicals used were purchased from Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

Preparation of crude enzymes. Procedures for crude enzyme preparation were carried out at 4 °C or on ice unless otherwise stated. Lemon peel (about 15 g fresh weight) were cut into small pieces and homogenized in 15 mL of 100 mM potassium phosphate (KPi) buffer (pH 6.5) containing 10 mM dithiothreitol (DTT), 2.5 mL of paraffin liquid, and 14% (w/v) polyvinylpolypyrolidone, using a polytron homogenizer (Kinematica, Lucerne, Switzerland). The homogenate was centrifuged at $9{,}200 \times g$ for 20 min, and the supernatant was desalted using a PD-10 column (GE Healthcare, Little Chalfont, UK) equilibrated with 100 mM Tris–HCl buffer (pH 7.5) containing 1 mM DTT. The eluted cell-free extracts (1.5 mL) were ultracentrifuged at $100{,}000 \times g$ for 30 min. The pellet obtained was resuspended in 750 µL of 100 mM Tris–HCl buffer (pH 7.5) containing 1 mM DTT using a dounce homogenizer (Wheaton, Millville, New Jersey), and was used as microsome fraction. The supernatant of ultracentrifugation was used as soluble fraction. Protein content was measured by the Bradford method with BSA as standard,[22] unless otherwise stated.

Enzyme assay. Cell-free extracts, the microsome fraction, and the supernatant fraction were used as crude enzymes. The standard incubation mixture consisted of the crude enzyme (35–45 µg), coumarin substrate (1.0 mM), prenyl substrate (1.0 mM), and $MgCl_2$ (10 mM) in a total volume of 200 µL. After incubation at 30 °C for 16 h, the reaction was stopped by the addition of 100 µL 3 N HCl. The reaction mixture was extracted with 300 µL of ethyl acetate containing 0.1 mM testosterone propionate as internal standard. The extract was dried and then dissolved in 50 µL of methanol. After centrifugation at $20{,}400 \times g$ for 5 min, 5 µL of the supernatant was used for HPLC analysis. In determination of optimum pH, the concentration of protein was measured with a Qubit® 2.0 fluorometer (Invitrogen, San Diego, California) following the manufacturer's protocol.

HPLC and LC-IT-TOF/MS analysis. Reaction products were quantified by HPLC (the D-2000 Elite HPLC System with an L-2455 photo diode array detector, Hitachi, Tokyo) using a LiChrosphere RP-18 column (4.0 mm × 250 mm, Merck, Darmstadt, Germany). A methanol:water:acetic acid (80:20:0.3) solvent was used at a flow rate of 1.0 mL/min. Separation was carried out at 40 °C. The detection wavelengths were 311 nm (products) and 254 nm (internal standard). Product identification was done by LC-IT-TOF/MS analysis, in which a TSK gel ODS-80Ts column (2.0 mm × 250 mm, Tosoh, Tokyo) was used, and the reaction products were ionized in positive ion mode. The detection range was from m/z 90 to m/z 510.

Results and Discussion

Detection of bergaptol 5-O-geranyltransferase (B5OGT) activity

To analyze the coumarin-prenyltransferase activities in the lemon fruit, we chose the outer part of the lemon peel (the flavedo) as enzyme source, because prenylated coumarin bergamottin accumulates in large quantities there.[10,23–25] The microsome fraction was prepared from flavedo and assayed for prenyltransferase activity using various substrate combinations. By use of bergaptol and GPP as substrates, we could detect a clear activity to synthesize bergamottin (Fig. 1A–C). Figure 1D shows various negative control reactions, incubation without bergaptol or GPP, incubation with EDTA (10 mM) instead of Mg^{2+}, incubation after inactivation of the crude enzyme at 95 °C for 20 min, and incubation without the crude enzyme. Bergamottin was not detected in any of the negative controls, indicating that the bergaptol 5-*O*-geranyltransferase (B5OGT) activity we detected was the native enzyme activity present in the lemon flavedo (Fig. 1C).

To clarify where exactly in the tissue of the lemon pericarp the biosynthesis of prenylated coumarins occurs, cell-free extracts of the flavedo and albedo, the white inner part of lemon peel, were prepared and used as crude enzymes. Only the reaction mixture containing the cell-free extract from the flavedo yielded bergamottin (Fig. 2), indicating that prenylated coumarins are biosynthesized in the flavedo and accumulate in the same tissue. After ultracentrifugation of the flavedo cell-free extract at $100{,}000 \times g$ for 30 min, the B5OGT activity was concentrated in the microsome fraction (Fig. 2), indicating that this enzyme is a membrane-bound protein, which is consistent with previously reported plant prenyltransferases specific to flavonoids.[13–15,26–28] The microsome fraction from the flavedo was then used as the crude enzyme in further analysis.

Enzymatic properties of B5OGT

First the linearity of B5OGT activity was checked. B5OGT activity did not decrease for 8 h (data not shown). Then kinetic analysis was performed within the reaction period under the conditions described in

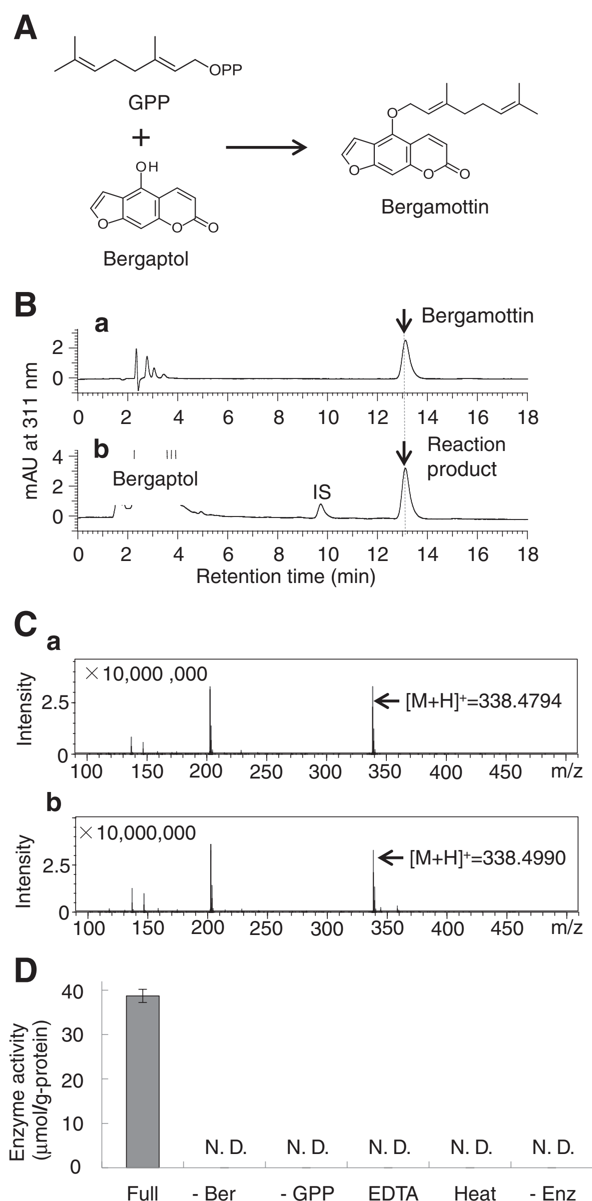

Fig. 1. HPLC Analysis of Enzymatic Reaction Products.

A, Bergaptol 5-*O*-geranyltransferase (B5OGT) reaction. B, HPLC chromatograms of reaction products detected at 311 nm. **a**, Standard specimen of bergamottin; **b**, reaction product of the microsome fraction of the lemon flavedo incubated with bergaptol and GPP in the presence of Mg^{2+}. Arrowheads indicate the retention times of bergamottin. IS represents the internal standard (testosterone propionate). C, Mass spectra of the reaction product on LC-IT-TOF/MS analysis. **a**, Standard specimen of bergamottin; **b**, reaction product of the microsome fraction of the lemon flavedo. D, Enzyme activities of full assay (Full) and various negative controls: incubation without bergaptol (-Ber), without GPP (-GPP), with 10 mM of EDTA instead of Mg^{2+}(EDTA), with denatured crude enzyme heated at 95 °C for 20 min (Heat), and with the incubation buffer instead of the crude enzyme (-Enz). Values are means ± SD for triplicate experiments. N.D., not detected.

"Materials and Methods." B5OGT showed an activity of $4.0 \pm 6 \times 10^{-3}$ µmol/h/g of protein on average in the flavedo of the mature lemon samples. The apparent K_m values were calculated by Hanes-Woolf plot to be 9 ± 2 µM for GPP and $1.4 \times 10^2 \pm 7$ µM for bergaptol (Fig. 3A). B5OGT showed high affinity for the prenyl substrate, comparable to the LePGT1 of *L. erythrorhizon*,[19] whereas B5OGT revealed low affinity for the aromatic substrate as compared to other plant mem-

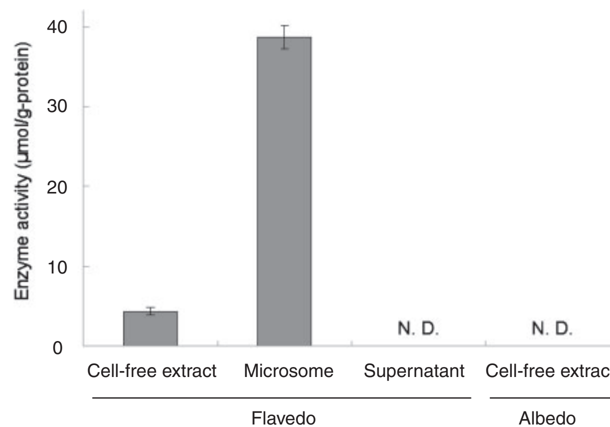

Fig. 2. Membrane Localization of B5OGT Activity.

The activities of various crude enzymes compared. Cell-free extracts prepared from the flavedo or the albedo of lemon peel, the flavedo microsome, and the supernatant after ultracentrifugation at $100{,}000 \times g$ for 30 min were used as crude enzyme preparations. N.D., not detected.

brane-bound prenyltransferases for the flavonoids reported so far.[13–15,26–28)]

The optimum pH was studied at pH 6.0–9.0 using KPi and Tris–HCl buffers. In agreement with previously reported coumarin-prenyltransferases, umbelliferone 6-prenyltransferase and umbelliferone 7-*O*-prenyltransferase of different plant species,[17,18] B5OGT showed a broad pH optimum of 7.5 to 8.0, and its activity decreased strongly in the acidic range (Fig. 3B). When MES-KOH buffer was used for the acidic range, lower activity than the KPi buffer was obtained, and at pH 5.0 no activity was detected (data not shown).

Divalent cations were absolutely required for B5OGT activity (Fig. 1D). Among the cations examined, Mg^{2+} was the most preferred (100%), followed by Co^{2+} (56%), Ni^{2+} (54%), Mn^{2+} (31%), Fe^{2+} (25%), and Zn^{2+} (8%) (Fig. 3C). Unlike previously reported coumarin-prenyltransferases,[16,18] the requirement for divalent cations of B5OGT was remarkably broad.

Substrate specificity

To analyze the substrate specificity of B5OGT activities in the lemon flavedo, various coumarins were used as prenyl acceptor substrates with DMAPP or GPP as prenyl donor in enzymatic reactions (Fig. 4A). When GPP was used as prenyl donor, the flavedo microsome fraction catalyzed the *O*-geranylation of bergaptol (100%), xanthotoxol (98%), and 5-hydroxy-7-methoxycoumarin (36%) to yield bergamottin, 8-geranyloxypsoralen, and 5-geranyloxy-7-methoxycoumarin respectively. Also, *C*-geranylation of umbelliferone (12%) yielding 8-geranylumbelliferone was detected (Fig. 4B). The reaction products were identified by direct comparison with standard specimens (Supplemental Fig. 1A–C; see *Biosci. Biotechnol. Biochem.* Web site), except for the geranylated product of 5,7-dihydroxycoumarin, for which a standard specimen was not available. In this case, the geranylated product was detected by LC-IT-TOF/MS (Supplemental Fig. 1D).

The *O*-geranylation activities and the geranylated products detected in this study are consistent with the prenylated coumarins that accumulate abundantly in the lemon flavedo, bergamottin, 8-geranyloxypsoralen, and

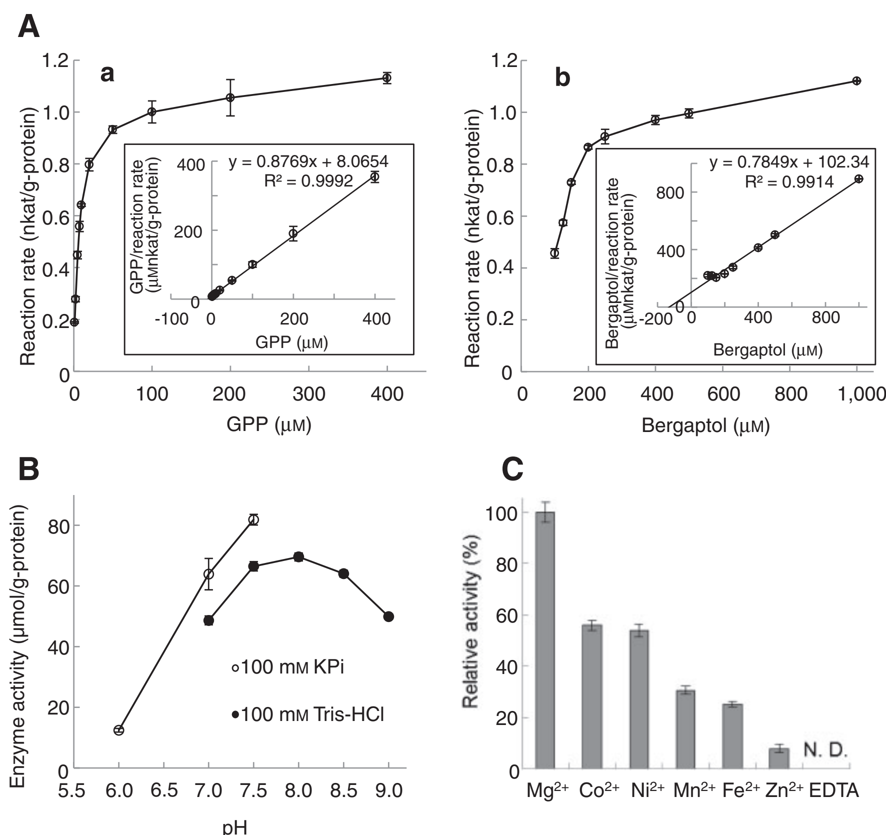

Fig. 3. Enzymatic Characterization of B5OGT.

A, Kinetic analysis of B5OGT. The reaction rate *versus* the concentration of GPP (a) and of bergaptol (b). Inserts indicate calculations of apparent K_m values by Hanes-Woolf plot using various concentrations of GPP (1.25–400 μM) and bergaptol (0.1–1 mM). B, pH dependency of B5OGT activity. B5OGT activity at various pH values was measured using 100 mM KPi buffer (at pH 6.0, 7.0, and 7.5) and 100 mM Tris–HCl buffer (at pH 7.0, 7.5, 8.0, and 9.0). C, Divalent cation requirement for B5OGT activity. A comparison of B5OGT activity in the presence of various divalent cations, Mg^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}. The activity in the presence of Mg^{2+} is shown as 100%. N.D., not detected.

5-geranyloxy-7-methoxycoumarin, *O*-geranylated compounds of bergaptol, xanthotoxol, and 5-hydroxy-7-methoxycoumarin respectively.[10,23–25] The *C*-geranylation activity of umbelliferone at the 8-position specifically found in the flavedo microsome fraction was unexpected, because 8-geranylumbelliferone has not been detected in the lemon. Further, this prenylation position at 8 leads to the formation of angular-type furanocoumarins, whereas, to the best of our knowledge, citrus fruits contain only linear-type furanocoumarins. While 6-prenylation of umbelliferone was not detected in the present study, this is the first report of geranylation for coumarin derivatives.

In contrast to GPP, DMAPP was not accepted as the prenyl substrate for the coumarin-prenyltransferases characterized in this study. Dimethylallylated coumarins, such as oxypeucedanin hydrate, imperatorin, and isoimperatorin, also accumulated in the lemon flavedo, but the contents were less than those of the geranylated coumarins.[10,24,25] The lemon flavedo might have low dimethylallyltransferase activities for coumarins, or, alternatively, the dimethylallylltransferase activities might have been more susceptible to degradation during the enzyme preparation procedures.

Prenylation of umbelliferone is the committed biosynthetic step in the formation of linear and angular furanocoumarins,[12] while DMAPP was not accepted as prenyl donor for umbelliferone in the lemon-peel enzyme. It is, however, possible that geranylumbelliferones also serve as precursors of furanocoumarin formation in the lemon, because the difference in chain length might not affect the oxidative carbon-carbon chain cleavage between C-3′ and C-4′ catalyzed by the cytochrome-P450 monooxygenase.[29,30] Thus the *C*-geranyltransferase activity detected in this study might be responsible for furanocoumarin biosynthesis in the lemon.

In summary, we detected both *O*- and *C*-geranyltransferase activities specific for coumarins in the lemon flavedo microsome fraction and performed a detailed characterization of B5OGT activity in the flavedo microsome. Our results indicate that coumarins were geranylated in the flavedo, where a large amount of geranylated coumarin derivatives accumulate, and that the enzymes responsible for prenylation were membrane-bound. The strongest activity detected was the *O*-geranyltransferase for bergaptol (B5OGT) to give bergamottin. B5OGT showed a broader divalent cation requirement than other plant prenyltransferases and had apparent K_m values for GPP, 9 ± 2 μM and for the coumarin substrate bergaptol, $1.4 \times 10^2 \pm 7$ μM. These findings provide information in the attempt to identify cDNAs encoding coumarin-specific prenyltransferases of the lemon. Genes coding for flavonoid prenyltransferases have been identified only in legume plants *e.g.*, *S. flavescens* (*SfN8DTs* and *SfG6DT*)[26,28] and the soybean (*GmG4DT*),[27] whereas a phloroglucinol prenyltransferase was recently cloned from hop (*Hl-PT1*).[31]

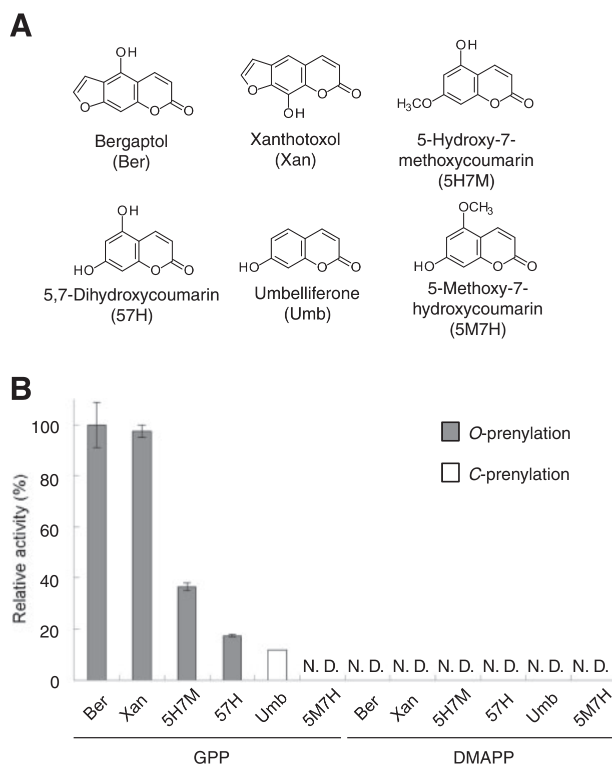

Fig. 4. Substrate Specificity of the Flavedo Microsome Fraction of Lemon Peel.

A, Chemical structures of coumarin substrates used in the enzyme assay. B, Comparison of enzyme activities with combinations of coumarin substrates and prenyl substrates (DMAPP and GPP). The quantity of geranylated 57H was calculated as the equivalent of geranylated 5H7M, because no standard specimen of geranylated 57H was available. Abbreviations for coumarins: Ber (bergaptol), Xan (xanthotoxol), 5H7M (5-hydroxy-7-methoxycoumarin), 57H (5,7-dihydroxycoumarin), Umb (umbelliferone), 5M7H (5-methoxy-7-hydroxycoumarin). N.D., not detected.

However, there has been no report on the identification of a prenyltransferase gene for coumarins and none on a *O*-prenyltransferase for aromatic substrates in plants. We assume that the *O*-prenyltransferases belong to a different enzyme family than the *C*-prenyltransferases, because all cloned aromatic prenyltransferases reported thus far show solely *C*-prenyltion activities. Bergamottin has drawn the attention of researchers due to its inhibitory activities against tumor cells[24] and oral bacteria that cause dental caries and periodontitis.[32] Identification of the *B5OGT* gene is hence expected in the future study.

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