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Ecology

Enzymatic activation of oleuropein: A protein crosslinker used as a chemical defense in the privet tree

(*Ligustrum obtusifolium*/iridoid glycoside/β-glucosidase/plant defense/glutaraldehyde)

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ABSTRACT **Leaves of the privet tree, *Ligustrum obtusifolium*, contain a large amount of oleuropein, a phenolic secoiridoid glycoside, which is stably kept in a compartment separate from activating enzymes. When the leaf tissue is destroyed by herbivores, enzymes localized in organelles start to activate oleuropein into a very strong protein denaturant that has protein-crosslinking and lysine-decreasing activities. These activities are stronger than ever reported from plant systems and have adverse effects against herbivores by decreasing the nutritive value of dietary protein completely. We report here that strong oleuropein-specific β-glucosidase in organelles activates oleuropein by converting the secoiridoid glucoside moiety of oleuropein into a glutaraldehyde-like structure, which is also an α,β-unsaturated aldehyde. Oleuropein activated by β-glucosidase had very strong protein-denaturing, protein-crosslinking, and lysine-alkylating activities that are very similar to, but stronger than, those of glutaraldehyde. Aucubin, another iridoid glycoside, had similar activities after β-glucosidase treatment. We also detected polyphenol oxidase activity in organelles that activate the dihydroxyphenolic moiety to have protein-crosslinking activities. These data suggest that the privet tree has developed an effective defense mechanism with oleuropein, a unique multivalent alkylator ideal as a protein-crosslinker. Our results that iridoid glycosides are precursors of alkylators may elucidate the chemical bases that underlie various bioactivities and ecological roles of iridoid glycosides.**

Recently, the importance of alkylating agents in plant–herbivore interactions has been recognized (1). Alkylating agents, which include quinones, epoxides, aldehydes, sesquiterpene lactone, pyrrolizidine alkaloids, etc., are diverse in their chemical structure, but they all have electrophilic atoms. These structures readily bind to various biological nucleophiles, including nucleophilic side chains of proteins (e.g., ε-NH_2 of Lys and -SH of Cys), and therefore exert adverse effects on herbivores (e.g., loss of nutritive value caused by loss of Lys and inactivation of enzymes), providing plants a chemical defense against herbivores (1–5).

The privet tree, *Ligustrum obtusifolium* (Oleaceae), is a small tree or a shrub that is widespread throughout East Asia and has been naturalized recently in the northeastern parts of the United States. Previously, we reported that the leaves of the privet tree have very strong protein-denaturing, protein-crosslinking, and lysine-decreasing activities that could be explained in terms of alkylating activities (6, 7). When protein is mixed with the leaf extract, protein denatures and forms a high-molecular-mass complex. At the same time, the lysine content of the protein decreases to one-third to one-fifth of the original, although other amino acids were not affected. As a result, the protein loses its nutritive value (6). Interestingly, addition of free glycine can inhibit all these activities (6, 7), and privet-specialist herbivores secrete free glycine in the digestive juice as an adaptation (6, 8). Purification study established that the compound responsible for the denaturing activity is oleuropein (Fig. 1), a phenolic secoiridoid glycoside found in several Oleaceae species such as the olive tree. Oleuropein makes up 3% of the wet weight of privet leaves (7). However, this compound itself is stable, does not have any of the activities, and is kept in the vacuoles or cytosol of the leaf cell. When the leaves are mechanically damaged by herbivory and cell compartments are broken, enzymatic activity localized in organelles separate from oleuropein starts to activate oleuropein into a very strong protein denaturant (7). Although the alkylating activities of privet leaves are stronger than those of other plants, the chemical mechanism of activation was not clear.

Iridoids (iridoid glycosides) are a group of terpene-derived compounds that have a common structure (9, 10). At present, almost 600 iridoids have been described from plants of 57 families (9) and are divided into three groups: (*i*) nonglycosidic iridoids, which have no sugar moiety, such as genipin (Fig. 1); (*ii*) iridoid glycosides, which typically have a single glucose molecule and a closed cyclopentane ring, such as aucubin and geniposide (Fig. 1); and (*iii*) secoiridoid glycosides, which also have a glucose molecule but no cyclopentane ring, such as oleuropein (9). Iridoids are known to have a variety of biological effects and have been implicated to play roles in plant–herbivore and prey–predator interactions (9, 10). As iridoids generally are toxic or deterrent to generalist herbivores, generalists usually experience a reduced growth rate or are deterred from feeding on iridoid-containing plants (9, 11, 12). However, iridoids have no negative effects on the feeding and growth of herbivores that specialize in feeding on iridoid-containing plants (9, 11). Some of these specialists sequester a large amount of iridoid (11, 13, 14) and have conspicuous warning coloration (13, 15). These insects are toxic and unpalatable to predators such as birds (15) and ants (16). Iridoids are also reported to have antimicrobial (17, 18), antitumor (19), hepatotoxic (20), bitter (8), and emetic features (15). Nevertheless, the chemical bases that lie under these interesting characteristics of iridoids have not yet been well explained.

In this study, we show that the activation of the secoiridoid glycoside moiety of oleuropein by substrate-specific β-glucosidase in organelles is crucial for oleuropein to exert the strong protein-denaturing/protein-crosslinking/lysine-alkylating activities in privet leaves. We also show that other iridoid glycosides could be similarly activated into alkylating agents by β-glucosidase and discuss the chemical bases for the biological activities of iridoids.

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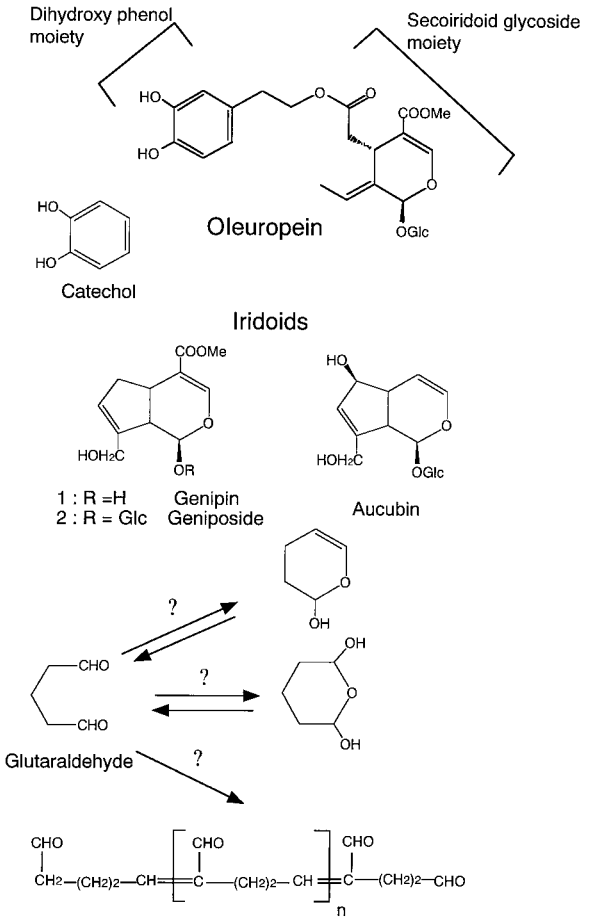

FIG. 1. Structures of oleuropein and its related compounds.

MATERIALS AND METHODS

Materials. Oleuropein was purified from the leaves of the privet tree (*Ligustrum obtusifolium*) as described (7). Aucubin was purchased from Nacalai Tesque (Kyoto). Geniposide, genipin, tropolone (polyphenol oxidase inhibitor), catechol, 20% (vol/vol) glutaraldehyde solution, ovalbumin, and *p*-nitrophenol were from Wako Pure Chemical Industries (Osaka). *p*-Nitrophenyl-β-glucopyranoside was from Senn Chemicals (Dielsdorf, Switzerland). β-Glucosidase (sweet almond) was purchased from Oriental Yeast (Osaka).

Preparation of Crude Enzyme Fraction (Organelle Fraction) from Privet Leaves. The enzyme fraction of the privet tree, which contains oleuropein-activating enzymes but not oleuropein, was prepared as described (7). In short, 16 g of fresh privet leaves were homogenized in 100 ml of high-osmotic-pressure sucrose solution (0.4 M sucrose/0.1 M sodium phosphate, pH 7) to collect intact organelles. After differential centrifugation and subsequent washing in the same buffer, collected organelles were resuspended in 20 ml of the same buffer. This suspension of intact organelles, where oleuropein-activating enzymes are localized, was used as the enzyme fraction. Breaking the organelles, a necessary step in assaying the enzyme activities inside them, was accomplished automatically when the organelles were burst in reaction solutions with low osmotic pressure.

Assays of Protein-Denaturing/Protein-Crosslinking/Lysine-Decreasing Activity. The activities were examined by testing whether ovalbumin would be denatured. All the assays were performed in 375 μl of 0.1 M sodium phosphate buffer (pH 7.0), containing 1% ovalbumin and 250 μl of 0 or 11 mM solutions of chemicals whose activities were to be measured (i.e., oleuropein, catechol, glutaraldehyde, geniposide, genipin, and aucubin). In trials designed to examine the effects of enzymes, 375-μl volumes of the reaction solutions also included 50 μl of the enzyme fraction of privet leaves or 50 μl of β-glucosidase solution, which contained 5 units of β-glucosidase (from sweet almond). Assays were performed in triplicate at 25°C for 2 h in 1.5-ml microfuge tubes with vigorous shaking. The effect of tropolone (polyphenol oxidase inhibitor; chelator of copper ion; refs. 21 and 22) on the denaturing activity was assayed by preincubating the 50-μl volumes of enzyme solutions with 90 μg of tropolone for 10 min before the initiation of the reaction; 5 μl of the reaction solution was applied to SDS/PAGE to determine the protein-denaturing/protein-crosslinking activities. The degree of denaturation and crosslinking was determined by the band pattern. For amino acid analysis, 1 ml of 6 M HCl and 700 μl of water were added to 300 μl of the reaction solution and hydrolyzed for 22 h at 110°C. After removal of HCl, lysine contents were analyzed with an auto amino acid analyzer (model L5000, Hitachi, Tokyo).

Assay of β-Glucosidase Activity and Assessment of the Degree of Deglucosidation. β-Glucosidase activity of the enzyme fraction (organelle fraction) on oleuropein, aucubin, and geniposide was assayed by measuring the amounts of glucose produced in the reaction solutions. To assay β-glucosidase activity of the enzyme fraction on oleuropein, an enzyme reaction was performed in triplicate in 375 μl of 0.1 M sodium phosphate buffer (pH 7.0) containing 250 ml of 11 mM solution of oleuropein, 10 μl of the enzyme fraction, and 90 μg of tropolone for 15 min at 25°C. Tropolone was preincubated with the enzyme fraction to inhibit the interference of polyphenol oxidase activity as described above. To assay β-glucosidase activity of the enzyme fraction on aucubin and geniposide, enzyme reactions were performed in 375 μl of 0.1 M sodium phosphate buffer (pH 7.0) containing 50 μl of the enzyme fraction and 250 ml of either 11 mM aucubin or geniposide at 25°C for 2 h. These conditions were within the linear range. Reactions were stopped by heat inactivation (96°C for 5 min). After centrifugation and filtration, 100-μl volumes of the supernatants from the reaction solutions were injected into an STR-ODS-H column (150 × 4.6-mm i.d., 5 μm; Shimadzu) to separate the produced glucose from chemicals that interfere with the detection of glucose. The column temperature was 40°C, and the flow rate was 1.0 ml/min. The gradient was as follows: 0–10 min, 0–70% (vol/vol) ethanol in water (linear gradient) and then 10–15 min, 70% ethanol (isocratic). A 1-ml volume of glucose-containing fraction of each sample was collected between 1.0 and 2.0 min, and the glucose concentration was analyzed by using GOD-PODLK (Nagase Biochemical, Kyoto), a glucose assaying kit based on a glucose oxidase-peroxidase-chromogen system, which uses phenol as a substrate of peroxidase and 4-aminoantipyrine as a chromogen (23, 24). Absorbance at 505 nm was measured. Glucose solutions of known concentrations in 0.1 M sodium phosphate buffer (pH 7.0) were also analyzed as standards by using HPLC and GOD-PODLK.

To assess the degree of deglucosidation of oleuropein and other iridoid glycosides by the enzyme fraction (organelles) or β-glucosidase, assays were performed in 375 μl of 0.1 M sodium phosphate buffer (pH 7.0) containing 11 mM solutions of an iridoid glycoside (oleuropein, geniposide, or aucubin) and 50 μl of the enzyme fraction of privet leaves or 50 μl of β-glucosidase solution, which contained 5 units of β-glucosidase (from sweet almond). In the reaction of oleuropein and the enzyme fraction of the leaves, the enzyme fraction was preincubated with tropolone as described above. Assays were performed in triplicate at 25°C for 2 h in 1.5-ml microfuge tubes with vigorous shaking. The glucose produced was analyzed as described above.

To assay the β-glucosidase activity of the enzyme fraction on *p*-nitrophenyl-β-glucopyranoside, a reaction was performed in

375 μl of 0.1 M sodium phosphate buffer (pH 7.0) containing 250 μl of 11 mM *p*-nitrophenyl-β-glucopyranoside, 90 μg of tropolone, and 50 μl of the enzyme fraction at 25°C for 2 h. After the reaction, 100 μl of the reaction solution was mixed with 2 ml of 1 M Na_2CO_3, and then absorbance at 450 nm was measured. The degree of deglucosidation was determined by comparing this absorbance with that of *p*-nitrophenol at 450 nm (25).

RESULTS

Protein-Denaturing/Protein-Crosslinking/Lysine-Decreasing Activities of Oleuropein: Activation of Phenolic Moiety by Polyphenol Oxidase. Oleuropein itself did not have any protein-denaturing and lysine-decreasing activities. There was no difference in SDS/PAGE pattern and lysine content between untreated ovalbumin and ovalbumin treated only with 11 mM oleuropein (Fig. 2 *A* and *B*, lanes 1 and 2). When organelles (the enzyme fraction), which have no protein-denaturing and lysine-decreasing activities (7), were added together in the mixture of ovalbumin and oleuropein, ovalbumin was denatured and high-molecular-mass crosslink products were formed, which are visible in the disappearance of the main band and in the appearance of fuzzy bands in the upper part of the stacking and separation gels (Fig. 2*A*, lane 3). At the same time, the lysine content of the treated protein decreased to approximately one-third of the original content (Fig. 2*B*, lane 3), but other amino acids were not affected (data not shown, but see refs. 6 and 7). These results suggest that activation by enzymes retained in organelles is necessary for oleuropein to exert its protein-denaturing/protein-crosslinking/lysine-decreasing activities.

To elucidate the chemical process of enzymatic activation of oleuropein, we first examined whether activation of the phenolic moiety is involved. The rapid browning that we observed when we mixed oleuropein and the enzyme fraction together, a hallmark of polyphenol oxidation and polymerization, supported the idea that the phenolic moiety is involved. Catechol (11 mM), a dehydroxyphenolic moiety of oleuropein itself, did not have any of the activities on ovalbumin without enzymatic activation (Fig. 2 *A* and *B*, lane 4). When mixed with the enzyme fraction (organelles) of privet leaves, 11 mM catechol had certain protein-denaturing/protein-crosslinking/lysine-decreasing activities (Fig. 2 *A* and *B*, lane 5) that were weaker than those observed when 11 mM of oleuropein was mixed with the enzyme fraction (Fig. 2, lane 3). The color of the reaction solution changed to dark brown. However, when we added 90 μg of tropolone (final concentration of 2 mM), a polyphenol oxidase inhibitor (21, 22), to the reaction between catechol and the enzyme fraction, all the activities and the browning of the reaction solution were inhibited completely (Fig. 2, lane 6). These data suggest that the enzyme fraction of privet leaves has a certain polyphenol oxidase activity that activates the phenolic moiety of oleuropein to some extent. However, contrary to our expectations, tropolone did not inhibit the protein-denaturing/protein-alkylating/lysine-decreasing activities of oleuropein activated by the enzyme fraction of privet leaves (Fig. 2, lane 7), although browning was inhibited completely. This result strongly suggested that some activation mechanism other than oxidation of the phenolic moiety exists and plays a greater role.

Protein-Denaturing/Protein-Crosslinking/Lysine-Decreasing Activities of Oleuropein: Activation of Iridoid Glycoside Moiety by β-Glucosidase. Next, we investigated the iridoid glycoside moiety and hypothesized that, if glucose is removed, after the ring-opening reaction in the hemiacetal-like structure and subsequent keto-enol conversion, the iridoid glycoside moiety may form a glutaraldehyde-like structure (Fig. 1). This conversion had been suggested to occur by several researchers (18, 20, 26), but its chemical, physiological and ecological consequences had not been studied in detail. As glutaraldehyde is well known as an potent alkylator, crosslinker, and denaturant of protein and is used to fix protein in histochemical studies (27–31), we hypothesized that the same reaction might occur in our system. To examine this possibility, we mixed 11 mM oleuropein with 5 units of β-glucosidase from sweet almond. Oleuropein incubated with β-glucosidase had protein-denaturing/protein-crosslinking/lysine-decreasing activities (Fig. 2 *A* and *B*, lane 8) just as strong as those observed when oleuropein was activated by the enzyme fraction of privet leaves in the presence of tropolone (Fig. 2, lane 7). Browning of the reaction solution did not occur

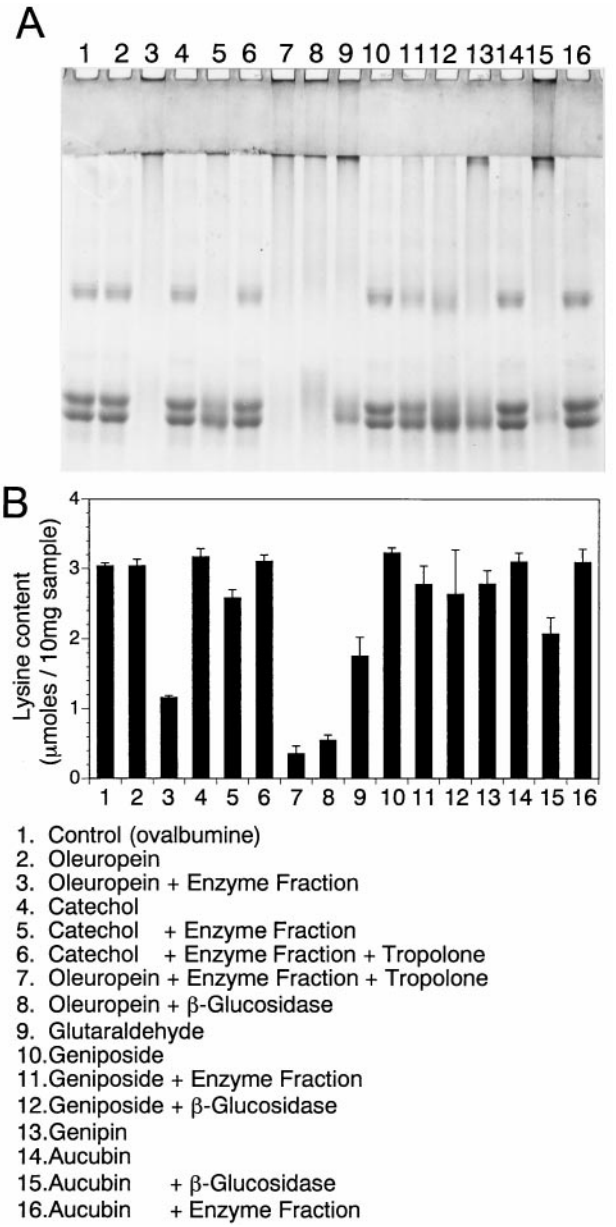

FIG. 2. Protein-denaturing/protein-crosslinking/lysine-decreasing activities of oleuropein, catechol, glutaraldehyde, and iridoid glycosides in the presence of the enzyme fraction of privet leaves (organelles), β-glucosidase, or tropolone (polyphenol oxidase inhibitor). (*A*) Protein-denaturing/protein-crosslinking activities on ovalbumin as assayed by SDS/PAGE. The degree of denaturation and crosslinking is indicated by the disappearance of the main band of ovalbumin and the appearance of fuzzy bands in the upper parts of stacking and separation gel. (*B*) Lysine-decreasing activity. After hydrolysis, the lysine content of ovalbumin in reaction solution was analyzed with an auto amino acid analyzer (Hitachi). Error bars represents SD ($n = 3$).

at all. We next examined whether glutaraldehyde has similar activities. Glutaraldehyde had activities (Fig. 2, lane 9) that are stronger than those observed in catechol activated by the enzyme fraction of privet leaves (Fig. 2, lane 5) but weaker than those observed in oleuropein activated by β-glucosidase (Fig. 2, lane 7). We further examined whether other iridoid glycosides are activated by β-glucosidase and by the enzyme fraction of privet leaves as well. Aucubin, which does not have any of the activities itself (Fig. 2 *A* and *B*, lane 14), had considerably strong activities when activated by β-glucosidase from sweet almond (Fig. 2, lane 15). The lysine-decreasing activity was as strong as that of glutaraldehyde (Fig. 2*B*, lanes 9 and 15), and the protein-denaturing/protein-crosslinking activities were stronger than those of glutaraldehyde and equaled those of oleuropein, as determined by the SDS/PAGE pattern (Fig. 2*A*, lanes 7–9 and 15). However, aucubin was not activated by the enzyme fraction of privet leaves (Fig. 2, lane 16). Geniposide, another iridoid glycoside that also has no activities itself, had weak activities after activation by β-glucosidase or by the enzyme fraction (Fig. 2 *A* and *B*, lanes 10–12). Geniposide is different from other iridoid glycosides in that its aglycone is relatively stable and is available as genipin. In agreement with this result, genipin had weak activities (Fig. 2, lane 13). All of these data indicate that oleuropein and other iridoid glycosides could be activated by β-glucosidase to exert activities very similar to those of privet leaves and glutaraldehyde.

β-Glucosidase Activity in the Enzyme Fraction Is Highly Specific to Oleuropein. To examine whether β-glucosidase really exists and plays a crucial role in privet leaves, we assayed β-glucosidase activity in the enzyme fraction (organelles) of privet leaves on oleuropein and other related β-glucosides (Table 1). The enzyme fraction (organelles) derived from 1 g of fresh privet leaves had 5.22 ± 1.01 μmol/min β-glucosidase activity on oleuropein. As 1 g of fresh leaves contains 55.5 μmol of oleuropein, the data show, theoretically, that the β-glucosidase activity is strong enough to deglucosidate and activate all the oleuropein in privet leaves in about 10 min. With geniposide as a substrate, the enzyme fraction from 1 g of fresh leaves had 0.142 ± 0.004 μmol/min β-glucosidase activity. With aucubin as a substrate, the same enzyme fraction had practically no activity (0.000 ± 0.000 μmol/min). With *p*-nitrophenyl-β-glucopyranoside, an artificial substrate often used in assaying β-glucosidase activity, as a substrate, this enzyme fraction had 0.029 ± 0.002 μmol/min activity. These data indicate that the enzyme fraction (organelles) of privet leaves has a strong and substrate-specific β-glucosidase activity on oleuropein and suggest that this β-glucosidase activity is the major factor that activates oleuropein in privet leaves.

Degree of Deglucosidation in Assaying Conditions. To interpret the results shown in Fig. 2 in greater detail, we examined the degree of deglucosidation in the assaying conditions by measuring the amounts of glucose produced after the reactions (Table 2). The enzyme fraction of privet leaves deglucosidated 92.2 ± 2.9% of oleuropein in the presence of tropolone (corresponding to lane 7 of Fig. 2). β-Glucosidase from bitter almond deglucosidated 40.9 ± 3.6% of oleuropein (Fig. 2, lane 8). The enzyme fraction of privet leaves deglucosidated 24.8 ± 0.7% of geniposide (Fig. 2, lane 11), and β-glucosidase deglucosidated 100.3 ± 12.9% of geniposide (Fig. 2, lane 12). Although β-glucosidase deglucosidated 34.4 ± 5.0% of aucubin (Fig. 2, lane 15), the enzyme fraction did not deglucosidate aucubin in assaying conditions (0.0 ± 0.0%; Fig. 2, lane 16). These enzyme assays based on the appearance of glucose showed good agreement with those based on the decrease of iridoid glycosides in the reaction solutions (as estimated by peak areas in HPLC) and also were in good agreement with those based on the appearance of genipin when geniposide was used (data not shown). In the case of oleuropein and aucubin, however, the corresponding aglycones were not observed in the reaction solutions as HPLC peaks, probably because of the instability of these aglycones. These data suggest that the inability of the enzyme fraction of privet leaves to activate aucubin is caused by its inability to deglucosidate aucubin and that the inability of the enzyme fraction to activate geniposide is caused by the inactivity of the corresponding aglycone. For an iridoid glycoside to exert strong activities, both the activeness of aglycone and the existence of efficient β-glucosidase are necessary. The oleuropein system of the privet tree seems to fulfill both criteria.

Table 1. β-Glucosidase activity in the enzyme fraction of the leaves of the privet tree (*Ligustrum obtusifolium*)

Substrate	β-Glucosidase activity, μmol/min/g leaf fresh weight*
Oleuropein	5.22 ± 1.01
Geniposide	0.142 ± 0.004
Aucubin	0.000 ± 0.000
p-Nitrophenyl-β-glucoside	0.029 ± 0.002

*Mean ± SD ($n = 3$).

Table 2. Percentage of deglucosidation of iridoid glycosides in conditions corresponding to those indicated in Fig. 2

Reaction	Deglucosidation*
Oleuropein + enzyme fraction† + tropolone	92.2 ± 2.9 (7)
Oleuropein + β-glucosidase‡	40.9 ± 3.6 (8)
Geniposide + enzyme fraction	24.8 ± 0.7 (11)
Geniposide + β-glucosidase	100.3 ± 12.9 (12)
Aucubin + β-glucosidase	34.4 ± 5.0 (15)
Aucubin + enzyme fraction	0.0 ± 0.0 (16)

*Mean ± SD ($n = 3$); The numbers in parentheses correspond to the lane numbers in Fig. 2.
†The enzyme fraction (organelle) of privet leaves.
‡β-Glucosidase from sweet almond.

DISCUSSION

This study chemically characterizes (*i*) the detailed mechanism of iridoid glycoside activation in an organism, (*ii*) the qualitative and quantitative chemical impact of activated iridoids on protein, and (*iii*) the unique multivalent protein crosslinker used as a chemical defense.

Our data clearly show that the privet tree is endowed with a very effective defense mechanism that uses oleuropein, a secoiridoid glycoside, as a defense chemical (Fig. 3). Leaves of the privet tree contain a large amount (3% of wet weight) of oleuropein, a precursor of a very active chemical species, compartmentally separated from activating enzymes. On attack by herbivores and possibly by pathogens, compartmentalization is broken and the activating enzymes β-glucosidase and polyphenol oxidase in organelles start to activate oleuropein into a very strong protein alkylator. We detected β-glucosidase activity that is enough strong to deglucosidate most of oleuropein in a short period of time (Table 1). Such compartmentalization with precursors and activating enzymes has been found in several plant chemical defense mechanisms (32) such as the cyanogenic glycoside-β-glucosidase system (33), the sinigrin-thioglucosidase system (34), and the phenolic-polyphenol oxidase system (35). Our finding of the iridoid glycoside-β-glucosidase system provides another example of the compartmentalization strategy of plant defense. In addition, β-glucosidase activity was found to be highly specific to oleuropein (Table 1). This result supports the idea that oleuropein and β-glucosidase have been developed as a set to provide a chemical defense against herbivores or pathogens. This kind of specificity is also found in the cyanogenic glycoside-β-glucosidase system (33). After activation, oleuropein has strong protein-denaturing/protein-crosslinking/lysine-

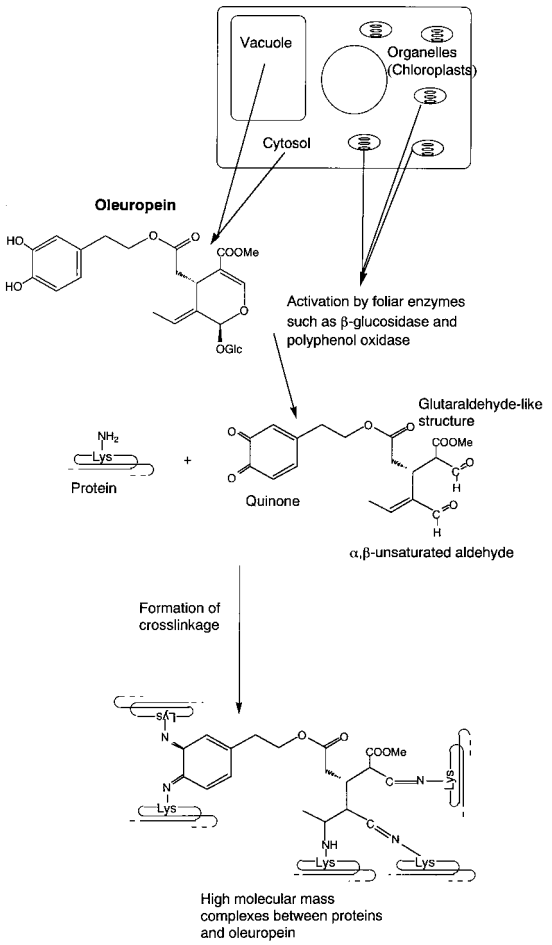

FIG. 3. Proposed chemical model of oleuropein activation in the privet tree, *Ligustrum obtusifolium*.

decreasing activities (Fig. 2), and our results suggest that these activities are caused by both the glutaraldehyde-like structure formed after deglucosidation of the iridoid glycoside moiety and the quinone structure formed after oxidation of the dihydroxy moiety of oleuropein. Glutaraldehydes and other aldehydes are potent alkylators and are known to bind to nucleophilic side chains of proteins (e.g., ε-NH_2 of Lys and -SH of Cys; refs. 1 and 27). Previous studies on protein fixation by glutaraldehyde show that glutaraldehyde binds intensively to amino residues in side chains of lysine in protein molecules and crosslinks protein molecules in these positions (27). These studies reported that the adduct formed between glutaraldehyde and amine is irreversible under acid hydrolysis, whereas the adduct formed between simple aldehyde and amine is reversible (27). Interestingly, the molecular structure of glutaraldehyde in aqueous solution and the chemical mechanism of the irreversible protein-crosslinking are still unclear and remain under debate (27–31), although several possible mechanisms have been proposed (see ref. 27 for review). Polymerization and isomerization of glutaraldehyde, which seem to occur through several types of reaction such as aldol condensation (Fig. 1), make it difficult to determine the exact structure of glutaraldehyde. However, the α,β-unsaturated aldehyde structures formed after the polymerization of glutaraldehyde (Fig. 1 *Bottom*) are said to be involved in the irreversible protein-crosslinking activity of glutaraldehyde, because amines easily bind to the β-carbon in α,β-unsaturated aldehyde through Michael addition and because the adduct is resistant to acid hydrolysis (27). Our observation that oleuropein activated by the enzyme fraction (Fig. 2, lanes 3 and 7) and by β-glucosidase (Fig. 2, lane 8) as well as glutaraldehyde (Fig. 2, lane 9) had strong protein-denaturing/protein-crosslinking/lysine-decreasing activities that are irreversible under acid hydrolysis is in good agreement with the previously reported chemical characteristics of glutaraldehyde (27) and with the idea that the glutaraldehyde-like structure was formed after the deglucosidation of the iridoid glycoside moiety of oleuropein. Quinones formed by oxidation of phenolics by oxidative enzymes, such as polyphenol oxidase and peroxidase, have been reported to have alkylating activity and to bind to nucleophilic side chains of proteins (e.g., ε-NH_2 of Lys and -SH of Cys; refs. 1–4). Our observation from the reaction of catechol and the enzyme fraction of privet leaves (Fig. 2, lane 5) is consistent with these reports. Furthermore, rapid and strong browning observed in oleuropein activated by the enzyme fraction suggests that the oxidation of the dihydroxyphenol moiety is indeed occurring. However, the fact that the activities of oleuropein activated by the enzyme fraction (Fig. 2, lane 3) were not inhibited by tropolone (Fig. 2, lane 7), which did inhibit the polyphenol oxidase activity in the enzyme fraction (Fig. 2, lanes 5 and 6), implies that activation in the secoiridoid glycoside moiety is of primary importance in privet leaves and that activation in the phenolic moiety is secondary. It is possible that the oxidized phenolic moiety (quinone) and the glutaraldehyde structure are competing for side chains of lysine. This idea—together with the observations that the adduct between lysine and glutaraldehyde is irreversible (27) and the adducts between lysine and oxidized phenolics are partly reversible (30–40%) to acid hydrolysis (4)—may explain why a greater lysine decrease is observed in the presence of tropolone (Fig. 2, lane 7) than in the absence of tropolone (Fig. 2, lane 3) when oleuropein was activated by the leaf enzymes. Further studies are needed to assign the role of the dihydroxyphenolic moiety in the activities of privet leaves.

We have previously shown that the lysine-decreasing activity of privet leaves adversely affects the growth of insects by decreasing the nutritive value of the food (6), because lysine is an essential amino acid and its absence limits insect growth. Recent studies showed that alkylating agents, such as oxidized phenolics, aldehydes, epoxides, and sesquiterpene lactones, have adverse effects on herbivores by binding to nucleophilic side chains of proteins (e.g., ε-NH_2 of Lys and -SH of Cys) and decreasing the nutritive value of the dietary protein (1–5). The antinutritive defense strategies of plants with such alkylating agents seem to be more common than previously expected. The privet tree system with iridoid glycoside and β-glucosidase seems to be one of the most obvious examples of the strategy. In accordance with our findings, only a small number of herbivorous species have adapted chemically to be able to feed on the privet tree (e.g., by secreting free glycine in digestive juice as a neutralizer; ref. 6).

The protein-denaturing/protein-crosslinking/lysine-decreasing activities after activation by β-glucosidase are not confined to oleuropein. Other iridoids, such as aucubin and geniposide, exerted similar activities. This finding suggests that the activation of iridoid glycosides by β-glucosidases into glutaraldehyde-like structures is a general phenomenon. Our result that iridoid aglycones have activities that are very similar to those of glutaraldehyde strongly suggests that the glutaraldehyde-like structure plays a crucial role in the activities of deglucosidated iridoids. In this sense, we can call iridoid glycosides "glutaraldehyde glycosides." Iridoid glycosides have been reported to have diverse biological activities, such as toxicity, sequestration, and unpalatability (9–20). These phenomena resemble the case of Monarch butterflies feeding on milkweeds, which contain cardenolides (36). Monarch butterflies sequester cardenolides, have warning coloration, and are unpalatable and emetic to birds. Cardenolides are inhibitors of Na^+/K^+-transporting ATPase and are heart toxins. In contrast, the chemical or physiological bases for the biological

activities of iridoid glycosides are still unclear. It has been observed *in vitro* that iridoid aglycones of aucubin, geniposide, and scandenoside methylester had antitumor activity (19) and that aglycone of oleuropein had antimicrobial activity (17, 18); however, iridoid glycosides (e.g., aucubin, geniposide, scandenoside, and oleuropein) did not have these activities (17–19). These observations indicate that iridoid glycosides have bioactivities after deglucosidation. Although there has been no clear indication of β-glucosidase activity from iridoid glycoside-containing plants except in our privet tree system, β-glucosidases have been found widely in animal tissues such as liver and kidney of vertebrates (37) and midgut tissues of insects (38, 39). Accordingly, it is possible that after ingestion, iridoids might be activated by intrinsic β-glucosidase of animal tissue into strong alkylators and may alkylate various cellular nucleophiles such as ε-NH_2 and -SH of proteins and nucleic acids, thereby exerting a variety of bioactivities such as toxicity (11, 12, 20). This idea may explain why geniposide was hepatotoxic in rats (20). Oleuropein and other iridoid glycosides are used as folk medicines (9), but they can be potential toxins that react with biomolecules after being deglucosidated by intrinsic β-glucosidases.

Our results show, however, that there are differences in the activities among the iridoids tested (Fig. 3). The activities of oleuropein were the strongest; those of aucubin were intermediate; and those of geniposide were the weakest. The strong activities of oleuropein could be caused by the α,β-unsaturated aldehyde structure that is expected after deglucosidation of oleuropein (Fig. 3). Double bonds in the α,β-unsaturated carbonyl structure are known to react with nucleophiles as in the case of drimane-type dialdehyde sesquiterpenes and sesquiterpene lactones (5). Also, it has been observed that the double bond stabilizes a bond formed between an aldehyde and an amino residue (27). The fact that oleuropein is a secoiridoid glycoside without a cyclopentane ring could be another reason for the strong activity of oleuropein. Iridoid aglycones are supposed to be in equilibrium between an open-ring glutaraldehyde-like structure and a closed-ring structure. In theory, the open-ring structure of oleuropein aglycone can rotate around two axes, whereas the open-ring structures of genipin and aucubin can rotate around only one. The free movement around two axes might favor the open-ring structure in equilibrium, which is an active form. The alkylating activities of iridoid glycosides might have correlations with biological activities. Interestingly, the antitumor activity of genipin is very weak and that of aucubin aglycone was relatively strong among several iridoid aglycones tested (19). This result might be in correlation with genipin having very weak activities on protein and aucubin having stronger ones (Fig. 2). Further chemical studies are needed to establish the correlations among molecular structures, alkylating activities, and biological activities of iridoid glycosides.

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