An Isoflavone Conjugate-hydrolyzing β-Glucosidase from the Roots of Soybean (Glycine max) Seedlings

PURIFICATION, GENE CLONING, PHYLOGENETICS, AND CELLULAR LOCALIZATION

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Soybeans (Glycine max (L.) Merr.) and certain other legumes excrete isoflavones from their roots, which participate in plant-microbe interactions such as symbiosis and as a defense against infections by pathogens. In G. max, the release of free isoflavones from their conjugates, the latent forms, is mediated by an isoﬂavone conjugate-hydrolyzing β-glucosidase. Here we report on the purification and cDNA cloning of this important β-glucosidase from the roots of G. max seedlings as well as related phylogenetic and cellular localization studies. The puriﬁed enzyme, isoﬂavone conjugate-hydrolyzing β-glucosidase from roots of G. max seedling (GmICHG), is a homodimeric glycoprotein with a subunit molecular mass of 58 kDa and is capable of directly hydrolyzing genistein 7-O-(6’-O-malonyl-β-D-glucoside) to produce free genistein (kcat 98 s⁻¹; Kₘ 25 μM at 30 °C, pH 7.0). GmICHG cDNA was isolated based on the amino acid sequence of the purified enzyme. GmICHG cDNA was abundantly expressed in the roots of G. max seedlings but only negligibly in the hypocotyl and cotyledon. An immunocytochemical analysis using anti-GmICHG antibodies, along with green ﬂuorescent protein imaging analyses of Arabidopsis cultured cells transformed by the GmICHG-GFP fusion gene, revealed that the enzyme is exclusively localized in the cell wall and intercellular space of seedling roots, particularly in the cell wall of root hairs. A phylogenetic analysis revealed that GmICHG is a member of glycoside hydrolase family 1 and can be co-clustered with many other leguminous β-glucosidasises, the majority of which may also be involved in ﬂavonoid-mediated interactions of legumes with microbes.

The roots of legumes excrete large amounts of ﬂavonoids, which play very important roles in the interactions of these plants with microorganisms (1). The major ﬂavonoids secreted from soybean (Glycine max (L.) Merr.) roots, for example, are isoflavones (2), a class of ﬂavonoids with a 3-phenylchromone structure. The isoflavones genistein and daidzein (see Fig. 1) in root exudates serve as chemoattractants (3) for speciﬁc symbionts, Bradyrhizobium japonicum and Sinorhizobium fredii, as well as genetic inducers of nodulation (4). Moreover, these ﬂavonoids have been proposed to play important roles in defensive mechanisms against infections by pathogens (5–9).

Isoflavones are synthesized from ﬂavanones (liquiritigenin or naringenin) by aryl migration, which is catalyzed by 2-hydroxy-ﬂavanone synthase (10, 11). In G. max cells, the resulting free isoflavones are then 7-0-glycosylated and 6”-O-malonylated by the combined actions of UDP-glucose:isoflavone 7-O-glycosyltransferase (IF7GT) and malonyl-CoA:isoflavone 7-O-glycosyl-6”-O-malonyltransferase (IF7MaT), respectively (5). These conjugates (i.e. 7-O-β-D-glucosides and 7-O-(6”-O-malonyl-β-D-glucosides) of isoflavones) are more water-soluble than the aglycons and accumulate in large amounts in vacuoles (5). These isoﬂavone conjugates are considered to be latent forms of isoﬂavonoids and must ultimately be converted to aglycons for interactions with symbiotic or pathogenic microorganisms (5, 7, 12). Moreover, the enzyme(s) involved in this conversion must be spatially separated from those of IF7GT and IF7MaT and the vacuolar conjugate pools (5). The release of aglycons in the chickpea (Cicer arietinum) has been proposed to involve a two-step hydrolysis of the isoﬂavone conjugates, where the isoﬂavone 7-O-(6”-O-malonyl-β-D-glucosides) undergoes ester hydrolysis by a malonylesterase followed by hydrolysis of the β-glucosidic linkage by a β-glucosidase (5). In G. max, however, the release of isoﬂavone aglycons from their conjugates appears to be mediated by a single enzyme, an isoﬂavone conjugate-hydrolyzing β-glucosidase (GmiCHG) (12) (Fig. 1), which appears to play a critical role in the turnover of conjugates for plant-microbe interactions. However, the cDNA encoding this important β-glucosidase has not been identiﬁed to date, and hence, the primary structure and phylogenetics of the enzyme

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† To whom correspondence should be addressed. Fax: 81-22-795-7270; E-mail: nakayama@seika.che.tohoku.ac.jp.
‡ The abbreviations used are: IF7GT, UDP-glucose:isoflavone 7-O-glycosyltransferase; FPLC, fast protein liquid chromatography; Gen7G, genistin 7-O-β-D-glucoside; Gen7MG, genistin 7-O-(6”-O-malonyl-β-D-glucoside); GmiCHG, ICHG from roots of G. max seedling; GH, glycoside hydrolase; HPLC, high performance liquid chromatography; ICHG, isoﬂavone conjugate-hydrolyzing β-glucosidase; IF7MaT, malonyl-CoA:isoflavone 7-O-glycosyl-6”-O-malonyltransferase; GFP, green ﬂuorescent protein; RT, reverse transcription; ER, endoplasmic reticulum.

2 The abbreviations used are: IF7GT, UDP-glucose:isoflavone 7-O-glycosyltransferase; FPLC, fast protein liquid chromatography; Gen7G, genistin 7-O-β-D-glucoside; Gen7MG, genistin 7-O-(6”-O-malonyl-β-D-glucoside); GmiCHG, ICHG from roots of G. max seedling; GH, glycoside hydrolase; HPLC, high performance liquid chromatography; ICHG, isoﬂavone conjugate-hydrolyzing β-glucosidase; IF7MaT, malonyl-CoA:isoflavone 7-O-glycosyl-6”-O-malonyltransferase; GFP, green ﬂuorescent protein; RT, reverse transcription; ER, endoplasmic reticulum.
Isoflavone Conjugate-specific β-Glucosidase of G. max

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Chemicals**

Soybean seeds (G. max (L.) Merr. cv. Wase-Hakuchô; Takii, Kyoto, Japan) were pretreated with running tap water for 10 min and then germinated at 20–23 °C in the dark on an agar medium consisting of 10 mM potassium Pi, pH 7.0, 1% (w/v) sucrose, and 0.5% (w/v) agar. Roots of 5–7-day-old seedlings were washed with tap water to remove the medium and then frozen at −80 °C until used. Genistein, Gen7G, Gen7MG, daidzein, daidzein 7-O-β-D-glucoside, and daidzein 7-O-(6-O-malonyl-β-D-glucoside) were products of Fujicco (Kobe, Japan). Other flavonoids, including anthocyanins, were obtained as described previously (13). All other chemicals were of analytical grade.

**Enzyme and Protein Assays**

GmICHG activity was routinely assayed using Gen7MG as a substrate. The standard reaction mixture (final volume, 100 μl) consisted of 50 μM Gen7MG, 100 mM potassium P, pH 7.0, and enzyme (final concentrations). The mixture was preincubated at 30 °C for 5 min and then incubated at 30 °C for 20 min, after which the reaction was stopped by heating to 80 °C. The reaction mixture was then passed through a 0.2 μm filter and used within 1 min. The isoflavonoids were detected by UV absorption at 260 nm using an SPD-10A VP UV-visible detector (Shimadzu, Kyoto, Japan). Kinetic parameters and standard errors were determined by fitting the initial velocity data to the Michaelis-Menten equation by means of a nonlinear regression analysis (14). Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard.

**Purification of GmICHG from Roots of G. max Seedlings**

All operations were performed at 4 °C.

**Step 1, Preparation of Crude Extract**—Soybean roots (200 g, fresh weight; see above) were suspended in 800 ml of 100 mM potassium Pi, pH 7.0, containing 30 mM 2-mercaptoethanol, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 5% (w/v) polyvinylpolypyrrolidone and were disrupted for 30 s in a Waring blender, followed by centrifugation. The supernatant was used for further purification.

**Step 2, Ammonium Sulfate Fractionation**—The protein fraction that precipitated between 0 and 60% saturation of ammonium sulfate was collected by centrifugation. The pellet was dissolved in 100 ml of 100 mM potassium Pi, pH 7.0, containing 30 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. Polyethylenimine was then added to the solution to a final concentration of 0.5% (w/v). After allowing the mixture to stand for 30 min, the precipitate was removed by centrifugation. The supernatant was extensively dialyzed against 10 mM potassium Pi, pH 7.0, containing 7.5 mM 2-mercaptoethanol (termed buffer A).

**Step 3, DEAE-Sepharose 4FF**—The enzyme solution was applied to a DEAE-Sepharose 4FF column (bed volume, 30 ml; Amersham Biosciences) equilibrated with buffer A at a flow rate of 2 ml/min using an FPLC system (Amersham Biosciences). The column was washed with the same buffer. The flow-through fractions that contained enzyme activity were collected.

**Step 4, CM-Sepharose 4FF**—The enzyme solution was applied to a CM-Sepharose 4FF column (bed volume, 20 ml; Amersham Biosciences) equilibrated with buffer A at a flow rate of 2.0 ml/min using an FPLC system. The column was washed with the same buffer. The enzyme was eluted with a linear gradient of 0–1 M NaCl in buffer B (20 mM potassium Pi, pH 7.0) in 100 min. The active fractions were combined.

**Step 5, Resource PHE**—Ammonium sulfate was added to the enzyme solution to 20% saturation. The enzyme solution was applied to a Resource PHE column (bed volume, 6 ml; Amersham Biosciences) equilibrated with buffer B containing 20%-saturated ammonium sulfate at a flow rate of 2 ml/min using an FPLC system. The column was washed with the same buffer. The enzyme was eluted at a flow rate of 2 ml/min with a linear gradient between the equilibration buffer and buffer B containing 50% (v/v) ethylene glycol in 30 min. The active fractions were combined and concentrated by ultrafiltration to 3.5 ml.
Step 6, Gel Filtration—The enzyme solution was applied to a HiLoad 26/60 Superdex 200pg column (Amersham Biosciences) equilibrated with buffer B containing 0.15 M NaCl. The enzyme was eluted at a flow rate of 1 ml/min using an FPLC system. The active fractions were combined and equilibrated with 10 mM potassium Pi, pH 7.0 (buffer C), by repeated concentration and dilution by ultrafiltration.

Step 7, HiTrap Heparin—The enzyme solution (2.5 ml) was applied to a HiTrap heparin column (1 ml; Amersham Biosciences) that had been equilibrated with buffer C at a flow rate of 0.5 ml/min using an FPLC system. The column was washed with buffer C containing 0.15 M NaCl. The enzyme activity was eluted with buffer C containing 1 M NaCl. The active fractions were combined, concentrated, equilibrated with buffer C, and subjected to rechromatography on a HiTrap heparin column in the same manner as described above.

Sugar Staining

Proteins in the SDS-polyacrylamide gels (16) were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The glycoprotein blots were visualized using methods reported previously (17), using a kit (G. P. Sensor, Seikagaku Corp., Tokyo, Japan) following the manufacturer's guidelines.

cDNA Cloning of GmICHG

Based on the amino acid sequences determined (see "Results"), degenerate oligonucleotide primers were designed as follows: PepF1, 5'-GGICCIAYATHTGGGAYAC-3'; PepP2, 5'-TTGGGAYACITTTTACAYA-3'; PepP1, 5'-TCCATRTACCAACCTATTGAYTGGT-3'; PepP2, 5'-CCRTAYTGRAARTCDATIGC-3', where I indicates inosine and R, H, and Y indicate degenerate sites (R, A/G; H, A/C/T; and Y, C/T). The total RNA was isolated from the G. max seedling roots using the RNeasy plant mini kit (Qiagen, Hilden, Germany). A reverse transcription-PCR (RT-PCR) was performed using Qiagen One-step RT-PCR kit (Qiagen, Hilden, Germany) at 37 °C for 30 min. For amplification of a 220-bp partial cDNA of GmICHG, a pair of gene-specific primers was designed as follows: GmICHG-S, 5'-CATAAAGGCTGGAAGGAGGTC-3'; GmICHG-A, 5'-TGGCTGTTGCTTGAGGAGGACAC-3'. For amplification of a 260-bp partial cDNA of G. max ubiquitin, a pair of gene-specific primers was also designed: GmUBQ-F, 5'-GGTTTTAAGCTCGTTGT-3'; GmUBQ-R, 5'-GGACACATTGTTTCAAC-3'. Semi-quantitative RT-PCR was carried out using mRNA Selective PCR kit (Takara bio, Tokyo, Japan) according to the manufacturer's guidelines.

Immunocytochemical Analyses

Cut roots (5–10 mm in length) of the 7-day-old G. max seedlings were fixed overnight at 4 °C in 20 mM sodium cacodylate, pH 7.4, containing 4% paraformaldehyde. After the fixed samples were washed extensively with 0.05 M potassium Pi, pH 7.5, they were embedded in 5% agar. The root sections (50-μm thickness) were prepared from the agar-embedded material with a Vibratome Series 3000 Plus-Tissue Sectioning System (Technical Products International, Inc.; St. Louis) using a razor blade. Immunocytochemical labeling of GmICHG was carried out using a kit (TSA kit 12 with horseradish peroxidase goat anti-rabbit IgG and AlexaFluor 488 tyramide; Molecular Probes, Eugene, OR) according to the manufacturer's guidelines. The resulting sections were observed under a laser scanning confocal microscope (Fluoview; Olympus, Tokyo, Japan) equipped with a filter set (BA5101F-BA550RIF).

Stable Transformation of Arabidopsis Cultured Cells and GFP Imaging

To construct the 35S-GmICHG:GFP construct, a full-length GmICHG cDNA fused in-frame with sGFP (S65T) (18), provided from Dr. Niwa (University of Shizuoka, Japan), was subcloned into the binary vector pBE2113Not (19). Arabidopsis thaliana T87 cultured cells (20) were transformed by co-cultivation with Agrobacterium tumefaciens GV3101(pMP90) carrying the resulting construct, as described in the Supplemental Material.

For GFP imaging, T87 cells expressing GFP-fused GmICHG were counterstained with a 10 μg/ml solution of propidium iodide (Sigma). The cells were observed under a BX50 microscope equipped with a confocal scanning system (Fluoview; Olympus, Tokyo, Japan). Green (sGFP) and red (propidium...
iodide and chlorophyll autofluorescence) emissions were detected with filters BA510IF-BA550RIF and BA585IF, respectively. The images were then pseudo-colored and combined into one image.

**RESULTS**

**Purification of GmICHG from the Roots of G. max Seedlings**—Our preliminary studies, as well as previous studies by Hsieh and Graham (12), showed that roots of the G. max seedling contain significantly higher (e.g. ~50-fold) ICHG activity than hypocotyls and cotyledons. Thus, we chose the roots of 5–7-day-old seedlings as the starting materials for the enzyme purification. The enzyme activity was highly stable throughout the purification procedures and was recovered in high yields at each purification step. Finally, a 4200-fold purification was achieved with an overall activity yield of 30% (Table 1). SDS-PAGE of the purified enzyme indicated a single major protein band with an estimated molecular mass of 58 kDa (Fig. 2A), which stained positive for the presence of sugar (Fig. 2B), indicating that GmICHG is a glycoprotein. The native molecular mass of the purified GmICHG was estimated to be 100 kDa by gel filtration chromatography on Superdex 200, indicating that the enzyme is dimeric. The N-terminal amino acid sequence (five cycles) of the purified enzyme, determined by automated Edman degradation, was Asp-Ser-Val-Pro-Leu-(sequence 3). To obtain the internal amino acid sequences of the purified protein, it was digested with a lysylendopeptidase from *Achromobacter lyticus* M497-1 (Wako, Tokyo, Japan), and the resulting peptides were separated by reversed phase HPLC as described previously (21, 22). The amino acid sequences of some of these peptides were determined to be as follows: Glu-Gly-Arg-Gly-Pro-Ser-Ile-Trp-Asp-Thr-Phe-Thr-His-Asn (sequence 2) and Ala-Ala-Arg-Ala-Ile-Asp-Phe-Gln-Tyr-Gly-Trp-Tyr-Met-Glu-(sequence 3).

**cDNA Cloning and Sequencing**—We designed the PCR primers on the basis of the amino acid sequences determined for the purified GmICHG (sequences 2 and 3, see supplemental Fig. 1S) and executed a first-round PCR using total RNA of the seedling roots (Fig. 2A) efficiently hydrolyzed both the malonlated and nonmalonlated forms of isoflavone 7'-O-β-d-glucosides, with the malonlated form being the preferred substrate. The *k*~cat~ and *K*~m~ values at pH 7.0, 30 °C for Gen7MG were 98 ± 3 s⁻¹ and 25 ± 2 μM, respectively, and those for Gen7G were 13 ± 1 s⁻¹ and 32 ± 2 μM, respectively (Table 2); thus, the calculated specificity constant (*k*~cat~/*K*~m~) for Gen7MG was ~10 times higher than the value for Gen7G. The 7'-O-(6'-O-β-d-malonyl-β-d-glucoside) and the 7'-O-β-d-glucoside of daidzein also acted as effective substrates, the specificity constants for which were 62 and 100, respectively.
Isoflavone Conjugate-specific β-Glucosidase of G. max

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity</th>
<th>k_{cat} (μM)</th>
<th>K_{m} (μM)</th>
<th>k_{cat}/K_{m} (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gen7MG</td>
<td>100</td>
<td>98 ± 3</td>
<td>25 ± 2</td>
<td>3.9</td>
</tr>
<tr>
<td>Gen7G</td>
<td>9</td>
<td>13 ± 1</td>
<td>32 ± 2</td>
<td>0.4</td>
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<tr>
<td>Dai7MG</td>
<td>64</td>
<td>46 ± 2</td>
<td>19 ± 2</td>
<td>2.4</td>
</tr>
<tr>
<td>Dai7G</td>
<td>17</td>
<td>22 ± 1</td>
<td>25 ± 2</td>
<td>0.9</td>
</tr>
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</table>

* The % activity was determined under the standard assay conditions (see "Experimental Procedures"), with the value of the substrate exhibiting the highest activity taken to be 100.

23% of the value for Gen7MG, respectively. The enzyme had negligible activity for the following glucosides (relative activity, less than 0.01% of activity for Gen7MG): cyanidin 3-O-β-d-glucoside, cyanidin 3-O-(6"-O-malonyl-β-d-glucoside), quercetin 3-O-β-d-glucoside, quercetin 3-O-(6"-O-malonyl-β-d-glucoside), p-nitrophenoxy β-d-glucoside, p-nitrophenoxy α-d-glucoside, and p-nitrophenoxy β-d-galactoside. Thus, the enzyme was highly specific for isoflavone conjugates.

Other enzymatic properties of the recombinant enzyme were also examined. The recombinant enzyme was successfully obtained as a soluble, catalytically active form, only when the mature form was expressed in E. coli cells as an N-terminal fusion protein with a thioredoxin molecule. The recombinant protein, 71 kDa in size, could be purified to homogeneity (Fig. 2S) in an activity yield of 22% (see Supplemental Material for details). It displayed a strong GmICHG activity that was highly specific for 7-O-β-d-glucosides and 7-O-(6"-O-malonyl-β-d-glucosides) of isoflavones, as is the case for the native GmICHG. Unlike the native GmICHG, however, the recombinant enzyme appeared to be unstable during purification and displayed lower k_{cat} values as well as a somewhat altered preference for isoflavonoids (see Table 1S). This could be related, at least in part, to the fact that the recombinant GmICHG was obtained as a fusion protein with a thioredoxin molecule and lacked sugar chain(s) in the molecule. The recombinant enzyme was active over the pH range 4.0–7.5 with a maximum activity at pH 5.5 and was stable at pH 6.5 (at 20 °C for 8 h). The enzyme activity was inhibited by 0.1 mM NaN₃ (residual activity, 1%). Other 0.1 mM metal ions (Ca²⁺, Co²⁺, Cu²⁺, Mg²⁺, Mn²⁺, Ni²⁺, and Zn²⁺) and 0.1 mM EDTA had negligible effects on catalytic activity (residual activity, in excess of 88%). The recombinant GmICHG was not inhibited by 0.1 mM glucono-δ-lactone, which has been reported to serve as a weak inhibitor for chickpea ICHG (23).

Sequence Comparison Studies—In an extensive search for sequence similarity in data bases and the literature, the highest identity was found with noncyanogenic β-glucosidases from chickpea (C. arietinum) (GenBank accession number CAG14979 (23)) and clover (Trifolium repens) (CAA40058 (24)) (identity, 69%), prunasin hydrolase from black cherry (Prunus serotina) (AAF34650 (25)) (64%), and dalcochinin (8'-O-β-glucoside β-glucosidase of Thai rosewood (Dalbergia cochinchinensis) (AAF04007 (26)) (64%), all of which are known plant members of the glycoside hydrolase (GH) family 1 (see Refs. 27 and 28). In the predicted amino acid sequence of the GmICHG precursor, we were consistently able to identify sequences that are ubiquitously found among the sequences of β-glucosidases belonging to GH1, such as Asn-Glu-Pro (residues 208–210) and Ile-Thr-Glu-Asn-Gly (residues 418–422) motifs (Fig. 1S), whose Glu residues are typical catalytic residues of GH1 enzymes (29, 30). Sugar-binding amino acid residues that are highly conserved among all GH1 enzymes (29, 30) could also be identified in the precursor sequence: Gln-59, His-163, Asn-208, Glu-209, Glu-420, Trp-469, Glu-476, and Trp-477. These results show that GmICHG is a member of the GH1 family.

Expression Analysis—The expression of GmICHG in G. max seedlings was analyzed by semi-quantitative RT-PCR. The GmICHG transcript was present in roots but was not detectable in either hypocotyl or cotyledon (Fig. 3). This is in very good agreement with the spatial distribution of enzyme activity in seedling (347 picokatal/mg protein in roots, 29 picokatal/mg protein in hypocotyls, and 16 picokatal/mg protein in cotyledons).

Localization Studies—To determine the cellular localization of GmICHG, we first used an immunocytochemical approach with antibodies (anti-GmICHG IgG) raised against synthetic peptides that are predicted to serve as epitopes of this enzyme (see Supplemental Material and Fig. 1S). Because the cotyledons and hypocotyls of G. max seedlings showed essentially no GmICHG activity and expressed only negligible levels of the GmICHG transcript (see above), we focused our analyses on the roots. A Western blotting analysis of crude extracts of G. max roots using anti-GmICHG IgG gave a single immunoreactive band (Fig. 4A), confirming its specificity for GmICHG.

In probing the cross-sections (Fig. 4B) and longitudinal sections (Fig. 4C) of root tissues of dark-grown G. max seedlings with anti-GmICHG IgG, immunopositive signals were found to be heterogeneously located in the peripheries of the cells, which likely correspond to the cell wall and intercellular space, in the epidermis, endodermis, and stele tissues of the root. It is noteworthy that many (but not all) of the cell corners as well as the middle lamella, which are known to be filled with pectic polysaccharides, showed strong immunopositive signals (Fig. 4,
In addition, GmICHG was abundantly expressed in root hairs (Fig. 4, D and E), in particular in the cell walls and apexes, although the expressed product appeared, in part, to remain in the cytoplasm of root hairs. Similar localization patterns were observed in root sections prepared from light-grown seedlings (data not shown).

To further confirm the localization of GmICHG in the plant cell wall, we introduced the GmICHG:GFP fusion gene into the T87 cultured cells of *A. thaliana*, and we observed the localization of the GFP signals in the transformed cells by means of confocal laser scanning microscopy. The results showed that the GFP signals arising from GmICHG-sGFP appeared to be mainly localized in the peripheries of the cells (Fig. 5, top panels), whereas the expression of sGFP without the fused GmICHG resulted in the localization of the GFP signals in the cytoplasm (Fig. 5, bottom panels). When the cells were counterstained with propidium iodide, which stains cell walls, the green fluorescence signals were co-localized with red fluorescence signals from the staining agent. Moreover, this pattern was not changed after plasmolysis of the transformed cells by treatment with 1 M mannitol (Fig. 5, middle panels), indicating that the expressed product is localized in the cell walls, and not in cell membranes, of the transformed cells. All of these results led us to conclude that GmICHG is localized in the cell walls and the intercellular space of *G. max* seedling roots.

**DISCUSSION**

GmICHG was purified from the roots of *G. max* seedlings to an extraordinarily high degree (4200-fold), and the primary structure, phylogenetics, and cellular localization of the enzyme were established, along with its molecular properties. The estimated subunit molecular mass of GmICHG was 58 kDa, not in agreement with values (80 and 75 kDa) estimated in a previous report (12). It is noteworthy that GmICHG

![Figure 4](http://example.com/image4.jpg)

**FIGURE 4.** Immunocytochemical analyses of the localization of GmICHG in the roots of the *G. max* seedling. A, SDS-PAGE analysis (lane 1) and Western blotting analysis using anti-GmICHG IgG (rabbit) (lane 2) of crude extracts of the *G. max* seedling roots. B–E, microscopic observations of the *G. max* seedling root, probed with a primary anti-GmICHG IgG (rabbit) and a secondary anti-rabbit IgG (goat)-peroxidase conjugate. B, confocal laser microscopic observations of a cross-section of a root. C, fluorescent microscopic observations of a longitudinal section of a root. D, confocal laser microscopic observations of root hairs. E, fluorescent microscopic observations of root hairs. Negative controls using preimmune antisera showed only negligible levels of fluorescence signals in the root tissues (not shown).

![Figure 5](http://example.com/image5.jpg)

**FIGURE 5.** Confocal laser microscopic observations of Arabidopsis T87 cultured cells transformed with the gene encoding an N-terminal fusion of sGFP with GmICHG (GmICHG:sGFP). Upper panels, T87 cells expressing GmICHG:sGFP. Granule-like red fluorescent signals represent autofluorescence from chloroplasts. Middle panels, T87 cells expressing GmICHG:sGFP were subjected to plasmolysis by treatment with 1 M mannitol and then stained with propidium iodide (PI), which is retained in the cell wall and emits a red fluorescence. Lower panels, T87 cells expressing sGFP were subjected to plasmolysis by treatment with 1 M mannitol and then stained with propidium iodide. The GFP is targeted to the cytoplasm. The cells were observed by confocal laser microscopy with filters BA510IF (left columns) and BA585IF (middle columns). The images obtained with each filter were then pseudo-colored and combined into one image (Merged, right columns). Bars, 20 μm.
Isoflavone Conjugate-specific β-Glucosidase of G. max

Symbiotic and defensive mechanisms

![Diagram of isoflavone conjugate pools in G. max](http://www.jbc.org/

FIGURE 6. Proposed enzymatic regulation of isoflavone conjugate pools in G. max. Dotted arrows indicate possible routes of translocation of isoflavonoids from the cytoplasm, via the vacuole, to the apoplast. Names of compounds are as follows: 1, flavanones; 2, isoflavone aglycones (R = H, daidzein; R = OH, genistein); 3, isoflavone 7-O-glucosides; and 4, isoflavone 7-O-(6′-O-malonyl-B-glucosides). IFS, 2-hydroxyflavanone synthase. A dimeric GmICHG molecule is shown in red. 2-Hydroxyflavanone synthase is a microsomal P450 enzyme (10, 11), whereas GmIF7GT and GmIF7MaT are predicted to be cytoplasmic enzymes. Isoflavone conjugates, 3 and 4, are considered to accumulate in vacuoles (5). GmICHG has been established to be an apoplastic enzyme (this study).

The results of immunocytochemical analyses, along with the GFP imaging analysis of Arabidopsis cells expressing GmICHG: GFP, conclusively show that GmICHG is localized in the cell wall and the intercellular space (collectively referred to as the apoplast) of seedling roots. The fact that the enzyme purified from the G. max roots is a glycoprotein is consistent with the known extracellular secretory pathway of proteins via ER-to-Golgi trafficking, although a PSORT analysis of the primary structure of the GmICHG precursor could not unambiguously predict such a sorting pathway. The pl value of the mature form of GmICHG is predicted to be 8.1, providing the possibility that this enzyme, a cationic protein, binds to pectic polysaccharides that are also present in plant cell walls and the intercellular space. It is noteworthy that GmICHG appeared to be heterogeneously distributed in the cell wall. This observation may be related to the possible heterogeneous distribution of materials that anchor this enzyme in the cell wall, or alternatively, it may arise from the restricted localization of secretory machinery for the enzyme precursor in root cells, resulting in the localized accumulation of the secreted enzyme in the cell wall.

The established apoplastic localization of GmICHG provides evidence for the spatial separation of GmICHG from isoflavone conjugate pools and other enzymes involved in isoflavonoid biosynthesis (Fig. 6). In G. max cells, isoflavone aglycons are formed by the action of 2-hydroxyflavanone synthase, an ER-localized P450 enzyme (10, 11). The resulting aglycons should then undergo 7-O-glycosylation and subsequent 6′-O-malonylation catalyzed by IF7GT and IF7MaT, respectively, to yield the conjugated forms (5). The roots of soybean seedlings also contain strong activities for these conjugating enzymes, both of which are predicted to be cytoplasmic enzymes (or located on the cytoplasmic surface of the ER). The resulting conjugates would then be transported to vacuoles and stored as latent forms to serve as large isoflavonoid pools that allow for isoflavone-mediated symbiotic or defensive mechanisms (5) (Fig. 6). When needed, these conjugates would be mobilized, probably in an exocytotic manner, from vacuoles to the apoplast, where they can be converted by the action of GmICHG to produce the biologically active free aglycons (Fig. 6). This compartmentation of GmICHG in the root apoplast is consistent with the role of this enzyme in the production of molecules that mediate interactions with soil microorganisms, because the root apoplast serves as an interface between root cells and the soil environment (1). For symbiotic interactions of G. max with rhizobia, free isoflavones, generated by the action of GmICHG in the root apoplast, need to be exuded into the rhizosphere, where they serve as specific chemoattractants for rhizobia to facilitate the establishment of accurate host-symbiont interactions. Most noticeably, it was observed that GmICHG was abundantly expressed in the cell walls of root hairs, ensuring the accumulation of large amounts of chemoattractants around root hairs. This is consistent with the fact that rhizobial infections are usually initiated by the specific adhesion of rhizobial cells to root hairs (1). During the course of the rhizobial invasion into the root interior, dividing rhizobial cells are entrapped within the invasion structure, termed the infection thread, which penetrates into the root cell layers (1). These processes are provoked by nod factors (4).

4 The estimated molar ratio of isoflavonoids extracted from roots of G. max seedlings was as follows: aglycons (genistein plus daidzein), 4%; 7-O-β-glucosides, 8%; and 7-O-(6′-O-malonyl-β-D-glucosides), 88%.
Isoflavone Conjugate-specific β-Glucosidase of G. max

FIGURE 7. Molecular phylogenetic tree of the amino acid sequences of the plant members of glycoside hydrolase family 1. The tree was constructed by the neighbor-joining method. Numbers indicate bootstrap values greater than 800. Leguminous β-glucosidases are shown in red. Clusters shown in color are those of GmICHG-related leguminous β-glucosidases (yellow; this study), disaccharide-specific β-glucosidases (light blue (35)), and thioglucosidases (gray (36)), respectively. Enzymes used for alignment are as follows: GmICHG, AB259819; dalcochinin β-glucosidase (D. cochinchinensis), AAF04007; β-glucosidase (C. arrietinum), CAG14979; noncyanogenic β-glucosidase (T. repens), CAA40058; β-glucosidase (A. thaliana), BAC42451; β-primeverosidase (Camellia sinensis), ABO88027; furcatin hydrolase (Viburnum fucatum), AB122081; prunasin hydrolase (P. serotina), AAF34650; amygdalin hydrolase (P. serotina), AA93234; furostanol glycoside 26-O-β-glucosidase (Costus speciosus), ABE79403; cardenolide 16-O-glucosidase (Digitalis lanata), AAB22162; cardenolide 16-O-glucosidase (Digitalis lanata), CAB38854; thioglucosidase (A. thaliana), CAA55787; myrosinase (Sinapis alba), CAA55787; myrosinase (Brassica napus), CAA42775; β-glucosidase (Zea mays), CAA52293; dhurrinase (Sorghum bicolor), AAC49177; α-glucosidase (Hordeum vulgare), AAC69619; raucaffricine-O-β-glucosidase (Rauvolfia serpentina), AAF03675; and strictosidine-O-glucosidase (Rauvolfia serpentina), CAC83098. GenBankTM accession numbers ABE79403, ABE83886, ABE85993, ABE85996, ABE86373, ABE86378, and ABE90582 are those of family 1 glycosidase hydrolases of Medicago truncatula.

which are lipo-oligosaccharides synthesized by rhizobia. The bacterial synthesis of nod factors is strongly induced by free isoflavones (4). Thus, it is plausible that the GmICHG-catalyzed production of free isoflavones in the apoplast of root endodermis facilitates an inward growth of infection threads as well as subsequent nodule morphogenesis in the root interior.

Sequence comparison studies revealed that GmICHG is related to the GH1 family, which includes β-glucosidases that play diverse and important roles in prokaryotes, Archaea, and eukaryotes. In higher plants, it has been proposed that GH1 β-glucosidases are involved in chemical defense against herbivores and pathogens (31), lignification (32), cell wall degradation (33), and regulation of the biological activity of phytohormones and other growth regulators by the hydrolysis of their inactive glucoside conjugates (34). A previous phylogenetic analysis of these plant GH1 members shows that enzymes sharing similarity in a specificity or biochemical role may be clustered with each other (35). The present analysis (Fig. 7) shows that GmICHG is closely related to β-glucosidases of legumes, such as Medicago, Cicer, Trifolium, and Dalbergia, many (but not all) of which appear to be categorized in a cluster that is separated from other plant β-glucosidases. Among these phylogenetically related leguminous β-glucosidases, dalcochinin β-glucosidase of D. cochinchinensis is the only enzyme that has been characterized biochemically (26), which hydrolyzes the β-glucosidic linkage of glucosides of an isoflavonoid (12-dihydromorphigenin), thus being similar to GmICHG in its substrate preference. It appears likely that members of this GmICHG-related cluster preferentially catalyze the degradation of flavonoid conjugates. Moreover, because the roots of legume plants are known to excrete flavonoid aglycons, the majority of the members of this cluster may also be involved in flavonoid-mediated symbiotic or defensive mechanisms, as in the case of GmICHG.

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