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Partial purification and characterization of a soybean β-glucosidase with high specific activity towards isoflavone conjugates

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Abstract

A β-glucosidase with high specific activity towards isoflavone conjugates was purified from soybean [*Glycine max*] roots by high salt extraction from a low speed centrifugal pellet and subsequent anion and cation exchange chromatography. Purification required stabilization throughout fractionation in 10% glycerol. The enzyme is most likely a dimer (approximate M_r 165 kDa) with potential subunits of M_r 80 and/or 75 kDa. The pH and temperature optima are pH 6 and 30 °C, respectively. The enzyme was highly heat-stable. Of the various potential effectors examined, silver and mercury ions were the most inhibitory. The IC_{50} of silver ions was increased from 140 μM to 14 mM in the presence of 250 μM β-mercaptoethanol. Glucono-δ-lactone was not strongly inhibitory (IC_{50} 24 mM). The activity was highly active against isoflavone conjugates, with a specificity constant 160–1000 fold higher for isoflavone conjugates over the generic chromogenic substrate, *p*-nitrophenyl β-glucoside. The enzyme was inactive against the flavonol glycosides tested. The partially purified enzyme had similar K_m and k_{cat} towards 7-*O*-glucosyl- and 7-*O*-glucosyl-6″-malonyl-isoflavones, suggesting that it may be able to cleave the esterified glucosyl conjugate. We hypothesize that the enzyme is involved in the release of daidzein and genistein, both of which play central roles in soybean defense.

Keywords: *Glycine max*; Leguminosae; Soybean; Phenylpropanoid; Isoflavone; Genistein; Daidzein; β-Glucosidase; Partial purification

1. Introduction

β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) comprise a heterogeneous group of enzymes that are able to cleave the β-glucosidic linkages of di- and/or oligo-saccharides, or other glucose conjugates. β-Glucosidases are widely distributed in the living world and play pivotal roles in many biological processes, such as degradation of cellulosic biomass, hydrolysis of glycolipids, cyanogenesis, and modification of secondary metabolites (Esen, 1993). In plants, β-glucosidase activity is involved in processes such as the compartmentalization and activity of phytohormones (Kleczkowski and Schell, 1995), defense mechanisms against microbes, insects, or parasitic plants (Bell, 1981; Poulton, 1990; Phillips and Streit, 1996), floral development and pigmentation (Harborne and Mabry, 1982; Koes et al., 1994), and they are also thought to have roles in lignification and cell wall decomposition (Hösel et al., 1978; Leah et al., 1995). Several β-glucosidases of plant origin are highly substrate-specific, such as those having selective specificity toward sapinin (Inoue and Ebizuka, 1996), hydrojuglone (Duroux et al., 1998), cinnamyl alcohols (Dharmawardhana et al., 1995), cyanogenic glycosides (Conn, 1993), flavones (Maier et al., 1993) and isoflavones (Hösel and Barz, 1975). Despite the availability of crystal structures for several plant β-glucosidases, the aglycone-binding site is still poorly understood. In a recent study, the active site of the maize β-glucosidase for the substrate DIMBOA-glucoside and its interaction with the cyanogenic glucoside dhurrin was examined (Czjzek et al., 2000).

Isoflavone conjugates are sometimes constitutively present in large quantities in legumes (see e.g. Graham,

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1991b). These isoflavone conjugates, including isoflavone 7-*O*-glucosides and isoflavone 7-*O*-glucoside-6″-*O*-malonates, may serve as pools for the release of free aglycones, which in turn may play important roles in plant-microbe interactions and defensive mechanisms against pathogen infection (Graham et al., 1990; Kessmann et al., 1990; Mackenbrock and Barz, 1991; Dakora and Phillips, 1996). Released isoflavones, for instance, can be either incorporated into the biosynthetic pathway of phytoalexins (Ebel, 1986; Barz and Welle, 1992; Kleczkowski and Schell, 1995) or act as preformed toxins, termed phytoanticipins (Johnson et al., 1976; Kramer et al., 1984; Weidenborner et al., 1990; Rivera-Vargas et al., 1993). Free isoflavones may also function as chemoattractants for both pathogens (Morris and Ward, 1992) and symbionts (Phillips et al., 1992) or as regulators of gene expression in Rhizobium spp. (Hungria and Stacey, 1997). More recently, the isoflavone genistein has been hypothesized to play a regulatory role in the activation of a soybean Type II NADH oxidase thought to participate in the establishment of competency for response to pathogen elicitors (see Graham and Graham, 1996a,b and reviews Graham and Graham, 1999, 2000).

In plants, the accumulation of isoflavone conjugates may be mediated by several enzymes (Fig. 1). The hydrolytic enzymes, isoflavone 7-*O*-glucoside-6″-*O*-malonate malonylesterase and isoflavone 7-*O*-glucoside β-glucosidase, from chickpea have been purified and characterized (Hösel and Barz, 1975; Hinderer et. al., 1986). Although non-specific β-glucosidase activities hydrolyzing isoflavone glucosides in the process of miso preparation and a lactase phlorizin hydrolase have been reported in soybean (Matsuura and Obata, 1993; Matsuura et al., 1995; Day et al., 2000), we know of no reports of characterization of β-glucosidases in soybean with preferential activity towards isoflavone conjugates. Because of the critical roles daidzein and genistein may play in soybean defense, we undertook the characterization of some of the enzymes involved in the turnover of their conjugates. In this report, a soybean isoflavone conjugate-hydrolyzing β-glucosidase (ICHG) was partially purified and characterized. The enzyme has high specific activity towards genistein/daidzein 7-*O*-glucoside (genistin and daidzin) and genistein/daidzein 7-*O*-glucosyl 6″-*O*-malonate (MGD and MGG), with very low K_m and high k_{cat} values. The specificity constant (k_{cat}/K_m) is 160–1000 fold higher for the isoflavone

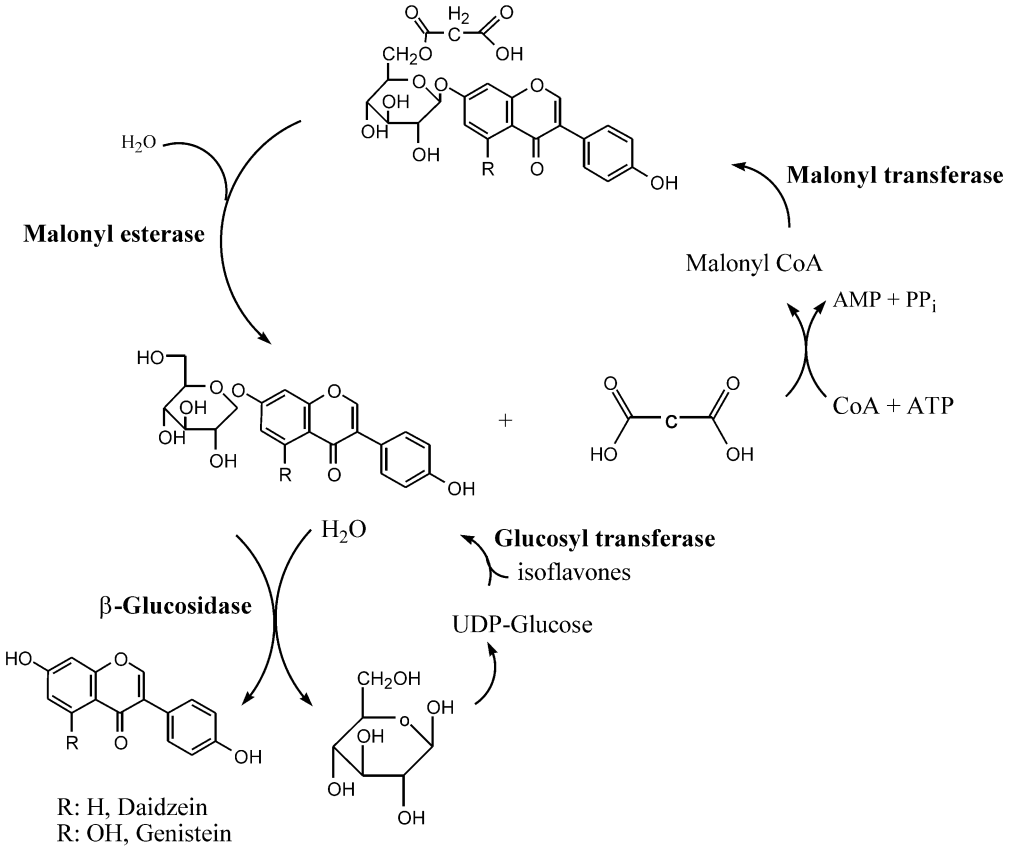


Fig. 1. Enzymatic regulation of the isoflavone conjugate pools. The scheme is based on the four purified enzymes from chickpea where malonylesterase and β-glucosidase hydrolyze isoflavone 7-*O*-glucoside-6″-*O*-malonates and isoflavone 7-*O*-glucosides, respectively, and glucosyltransferase and malonyltransferase transfer glucose and malonate sequentially to the isoflavone moiety.

conjugates over the chromogenic substrate *p*-nitrophenyl β-glucoside. Isoflavone conjugate hydrolysis in soybean may differ from that of chickpeas in that we found no evidence for an esterase active on MGG or MGD. Although we cannot preclude the existence of such an esterase, the partially purified glucosidase characterized in this report was able to hydrolyze both the glucosyl and malonyl glucosyl isoflavones directly with similar kinetics.

2. Results and discussion

2.1. Purification of the isoflavone conjugate-hydrolyzing β-glucosidase

Many studies were performed with various soybean organs to develop the optimal purification protocol. For example, vacuum infiltration of soybean cotyledons with 0.2 M phosphate buffer, pH. 6, containing 200–500 uM NaCl and subsequent isolation of the intercellular washing fluids by centrifugation led to a preparation highly enriched for the glucosidase, suggesting a possible apoplastic localization. These intercellular washing fluids contain no cytoplasmic marker enzymes (T.L. Graham, unpublished). However, due to the relatively low level of the ICHG in cotyledons and the difficulty in preparing large amounts of the intercellular fluids, we chose to work with roots. Roots contained the highest specific activity of ICHG in the soybean seedling. For example, a crude preparation from roots was about 800-fold more enriched for the enzyme than a crude preparation from cotyledons (data not shown). In roots, activity was found to be associated with the low speed centrifugal pellet.

Enzyme activity was solubilized from the pellet with 0.2 M phosphate buffer, pH 6, containing 0.5 M NaCl and fractionated by 40–60% ammonium sulfate saturation. Due to empirical results regarding the instability of the enzyme, a desalting column (Sephadex G-25) was chosen over dialysis for salt removal, and 10% glycerol was always present in the buffer from this step onward. When the desalted protein solution was passed through a DEAE-Sephadex A-50 column at pH 6.0, nearly all of the enzyme activity was in the unbound fractions (Fig. 2A). The active fractions were pooled and 5 mM final concentration of sodium ascorbate was introduced to maximize stability. The protein sample was then loaded onto a CM-Sephadex C-50 column and eluted with a linear gradient of buffer F in buffer E. The activity peak was eluted at 0.56 M NaCl (Fig. 2B). SDS polyacrylamide gel electrophoresis (PAGE) of various fractions under reducing conditions is shown in Fig. 3. Although the banding patterns for the fractions from the DEAE and CM-Sephadex columns are qualitatively similar, the analysis of the gel with ImageQuant software (Molecular Dynamics) determined that the pixel volume of the 80 kD band was three to four fold enriched in comparison to the other minor bands. This is consistent with the separation from a majority of other proteins for this fraction as shown in Fig. 2B. At this point, the ICHG was purified approximately 20-fold with a 20% recovery rate (Table 1). The most active fractions were pooled and lyophilized until almost only glycerol was left. Aliquots of this glycerol solution of ICHG were stored at −20 °C for further study. Under these conditions, the enzyme aliquots did not freeze solid and activity was stable for at least six months.

2.2. Properties of the isoflavone conjugate-hydrolyzing β-glucosidase

The molecular mass of ICHG upon SDS PAGE under non-reducing conditions was judged to be approximately 165 kDa (data not shown), while under reducing conditions a major band of mass 80 kDa and a less intensely staining band of 75 kDa were seen (Fig. 3). Though both of these bands were reproducibly associated with activity throughout purification, it is not clear whether the two bands represent different polypeptides or different post-translational modifications of a single polypeptide. Thus, while it is likely that the enzyme is a dimer, with monomers linked through a disulfide bond, its precise subunit composition will require further clarification. The ICHG tolerates very high concentrations of β-mercaptoethanol, with 50% inhibition of enzyme activity (IC_{50}) at 1.1 M, suggesting that the monomer form may also be catalytically active. Many β-glucosidases contain either identical or non-identical subunits. The number of subunits varies from 2 to 10 (or more) depending on the enzyme. For instance, a 60 kDa coniferin-hydrolyzing β-glucosidase from lodgepole pine has two non-identical subunits with M_r of 28 and 24 kDa (Dharmawardhana et al., 1995). The furostanol glycoside 26-*O*-β-glucosidase from *Costus speciosus* is a 110 kDa protein with two subunits with M_r of 54 and 58 kDa (Inoue and Ebizuka, 1996). Furthermore, the isoflavone hydrolyzing β-glucosidase from chickpea has apparent M_r of 125–135 kDa with two identical subunits of 68 kDa (Hösel and Barz, 1975).

The ICHG showed a pH optimum at approximately pH 6 at 30 °C. The optimum temperature was 30 °C. Both are typical for β-glucosidases (Esen, 1993). The enzyme is highly heat stable and remains substantially active (87%) after 45 min at 50 °C.

Several chemicals reported to be effectors of β-glucosidases were examined. Of all the chemicals tested for the inhibition of enzyme activity, only silver and mercury ions were inhibitory at the 1 mM level (Table 2), suggesting that sulfhydryl groups may play an essential role in enzyme activity. However, other sulfhydryl

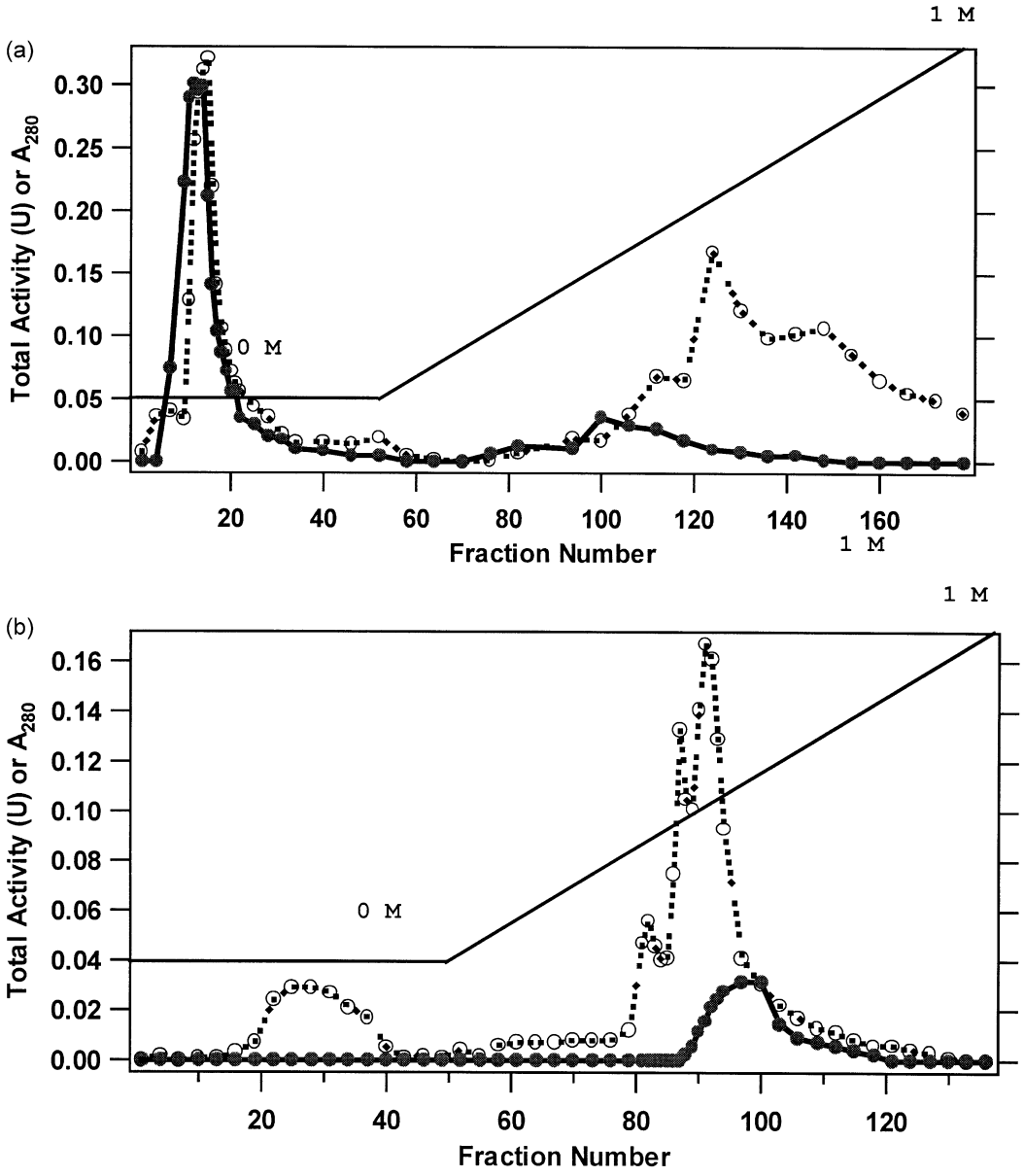


Fig. 2. The purification of the isoflavone-specific β-glucosidase. The chromatograms for anion exchange on DEAE-Sephadex (a), and cation exchange on CM-Sephadex (b). Symbols: open circle and dashed line, A280; closed circle and solid line, total activity. The linear salt gradients are shown.

Table 1
The purification scheme for the isoflavone conjugate-hydrolyzing β-glucosidase from soybean roots

Purification step	Total activity[a] (mU)	Total protein (mg)	Specific activity (mU/mg protein)	Yield (%)	Purity (fold)
Root cell wall wash	8780	124	70	100	1
Ammonium sulfate (40–60%)	10,540	50	220	120	3
DEAE-Sephadex	2960	12	240	34	3.4
CM-Sephadex	1740	1.3	1340	20	20

[a] The enzyme activity was measured by an HPLC method using a natural isoflavone conjugate mixture as substrate. The activity was calculated from the concentration of the released daidzein.

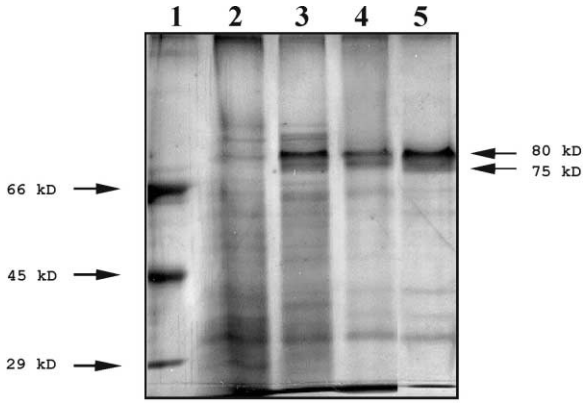


Fig. 3. SDS-PAGE analysis. Protein samples from each step of purification were subjected to SDS-PAGE (7.5% acrylamide) under reducing conditions. lane 1, protein markers; lane 2, crude root pellet extract; lane 3, 40–60% ammonium sulfate fraction; lane 4, activity peak from DEAE-Sephadex; lane 5, activity peak from CM-Sephadex.

group modifiers such as iodoacetate and *p*-chloromercuribenzoate (PCMB) had little or no effect (Table 2), possibly due either to the different accessibility of the protein SH groups to these compounds (Esen, 1992) or to their different redox potentials (Jocelyn, 1972). Consistent with the presence of critical sulfhydryl groups, inhibition by silver ions was prevented in the presence of β-mercaptoethanol. In the presence of 250 mM β-mercaptoethanol, the IC_{50} for Ag^{+} inhibition increased 100 fold from 140 μM (without β-mercaptoethanol) to 14 mM (data not shown). The importance of SH groups in ICHG is consistent with the fact that cysteine residues are involved in stability and enzyme activity of β-glucosidases (Rotrekl et al., 1999; Hakulinen et al., 2000), although most β-glucosidases in Family 1 (Henrissat, 1991) require two essential carboxylates for their catalytic activity, contributing to the general acid/base and nucleophilic catalysis (Zechel and Withers, 2000). The transition state analogue inhibitor, glucono-δ-lactone, was not very effective. The IC_{50} with this inhibitor was 24 mM (data not shown), while the counterpart in chickpea was more sensitive to this inhibitor, with an IC_{50} of 1 mM (Hösel and Barz, 1975).

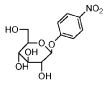
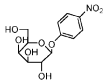
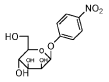
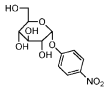
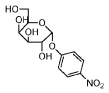
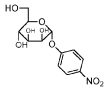
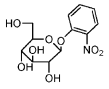
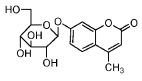
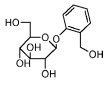
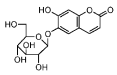
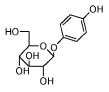
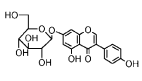
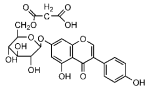
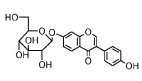
The activity of the ICHG on a variety of glycoside substrates is summarized in Table 3. This enzyme is able to hydrolyze a range of synthetic glucosides, but generally only with relatively low specificity constant (k_{cat}/K_m). Although the enzyme has moderate affinity with several other natural glucosides, the specificity constant for those tested is far lower than that of the isoflavone conjugates (Table 3). Interestingly, the enzyme showed no measurable activity with several flavonol glycosides, although we did not test the 7-*O*-substituted flavones or flavonols. In contrast, commercial almond emulsin β-glucosidase has much higher specificity toward the synthetic substrate, *p*-nitrophenyl β-glucoside than toward the isoflavone conjugates (Table 3). While almond emulsin β-glucosidase does not hydrolyze the malonylglucosyl isoflavone conjugates, consistent with the fact that most β-glucosidases only cleave non-modified terminal glucose residues, the partially purified ICHG apparently hydrolyzes malonyl isoflavone glucosides as well as isoflavone glucosides with similar kinetics (Table 3). The breakdown of isoflavone conjugates is mediated by two distinct enzymes in chickpea, a malonylesterase and a β-glucosidase, which successively

Table 2
The effect of inorganic and organic chemicals on the activity of the isoflavone-conjugate hydrolyzing β-glucosidase

Inorganic chemicals		Relative activity[a] (%)	Organic chemicals[b]		Relative activity[a]
$AgNO_3$	1 mM	35	Iodoacetate	1 mM	97
	10 mM	0		10 mM	74
$HgCl_2$	1 mM	62	PCMB	0.1 mM	102
	10 mM	23		1 mM	103
$CaCl_2$	1 mM	99	Glucono-δ-lactone	1 mM	99
	10 mM	94		10 mM	93
$MgCl_2$	1 mM	92	PMSF (in 95% EtOH)	1 mM	89
	10 mM	82		control	97
$CoCl_2$	1 mM	97		10 mM	41
	10 mM	102		Control	37
$MnCl_2$	1 mM	99	Eserine	1 mM	87
	10 mM	74	DMSO	5%	117
$CuSO_4$	1 mM	99	EGMME	5%	109
	10 mM	115	EGDME	5%	120
$ZnSO_4$	1 mM	104			
	10 mM	77			

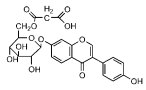
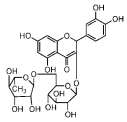
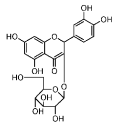
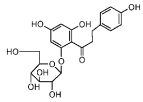
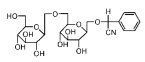
[a] Values are the means of two determinations in two independent experiments.
[b] Chemical abbreviations: PCMB, *p*-chrolomercuribenzoate; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; EGGME, ethylene glycol monomethyl ether; EGDME, ethylene glycol dimethyl ether.

Table 3
Reaction parameters and substrate specificity of the isoflavone conjugate-hydrolyzing glucosidase (ICHG) compared with that of the glucosidase from almond emulsin (AEG)

Substrates	Structures	ICHG			AEG		
		K_m[a] (M)	k_{cat}[a] (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m[a] (M)	k_{cat}[a] (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
p-Nitrophenyl β-glucoside		1.3×10^{-3}	0.11	8.1×10^{1}	2.2×10^{-3}	1.2×10^{2}	5.2×10^{4}
p-Nitrophenyl β-galactoside		1.3×10^{-2}	0.93	7.2×10^{1}	2.6×10^{-3}	6.8	2.6×10^{3}
p-Nitrophenyl β-mannoside		N.R.[b]	–	–	N.R.	–	–
p-Nitrophenyl α-glucoside		N.R.	–	–	N.R.	–	–
p-Nitrophenyl α-galactoside		8.4×10^{-4}	0.49	5.8×10^{2}	N.R.	–	–
p-Nitrophenyl α-mannoside		9.9×10^{-3}	0.10	1.0×10^{1}	[c]	–	–
o-Nitrophenyl β-glucoside		1.4×10^{-3}	1.4	1.0×10^{3}	3.9×10^{-3}	5.0	1.3×10^{3}
4-Methylumbelliferyl β-glucoside		2.3×10^{-4}	0.05	2.3×10^{2}	2.3×10^{-3}	4.7×10^{1}	2.0×10^{4}
Salicin		3.5×10^{-3}	0.21	6.0×10^{1}	1.1×10^{-2}	0.83	7.6×10^{1}
Esculin		8.8×10^{-3}	0.02	2.8			
Arbutin		6.1×10^{-3}	0.15	2.4×10^{1}			
Genistein 7-*O*-glucoside (genistin)		3.3×10^{-4}	4.45	1.3×10^{4}	1.1×10^{-2}	0.65	5.9×10^{1}
Genistein 7-*O*-glucosyl 6-*O*-malonate (MGG)		5.4×10^{-5}	4.38	8.1×10^{4}	[c]	–	–
Daidzein 7-*O*-glucoside (daidzin)		9.1×10^{-5}	3.07	3.4×10^{4}			

(*continued on next page*)

Table 3 (*continued*)

Substrates	Structures	ICHG			AEG		
		K_m[a] (M)	k_{cat}[a] (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m[a] (M)	k_{cat}[a] (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Daidzein 7-*O*-glucosyl 6"-*O*-malonate (MDG)		1.2×10^{-4}	4.34	3.6×10^{4}			
Rutin		N.R.	–	–			
Isoquercitrin		N.R.	–	–			
Phloridzin		N.R.	–	–			
Amygdalin		[c]	–	–			

[a] Values are means of determinations in three independent experiments.
[b] N.R.: no reaction at 40 °C for 30 min.
[c] The products were too small to measure or difficult to calculate K_m and V_{max}.

remove the malonyl and glucose moieties, respectively (Fig. 1). By using the isoflavone conjugate mixture coupled with the HPLC assay, we are able to examine these two enzyme activities simultaneously. However, a separate malonylesterase activity was never observed in any soybean tissue extract or at any stage of purification. Moreover, the lack of esterase activity in the partially purified IGHG preparation was also demonstrated when malonylated isoflavone glucosides were the sole substrates in the assay system with an excess of the glucosidase inhibitor, glucono-δ-lactone, over its IC_{50} value (data not shown). While the current data certainly do not preclude the existence of a separate isoflavone malonyl esterase in soybean and do not unequivocally support the existence of both activities in ICHG, it is possible that the soybean system may differ in this regard from the chickpea system. In the literature, only one other β-glucosidase, the flavone 7-*O*-glucoside-specific β-glucosidase from ligulate florets of *Chamomilla recutita*, can also use apigenin 7-*O*-acetylglucoside as a substrate, but the V_{max} was reduced dramatically when the glucose is esterified (Maier et al., 1993). Ultimately, more detailed characterization of the various enzymes will be needed to resolve these issues.

3. Conclusions

The hydrolysis of glucosyl and malonylglucosyl isoflavone conjugates is biologically relevant. A relatively high percentage of the total malonylated isoflavone glucosides is found in the apoplast in soybean (T. L. Graham, unpublished observation) and in chickpea. The genistein aglycone is toxic to fungal pathogens, such as *Phytophthora sojae* (Rivera-Vargas et al., 1993), and genistein also may function to activate an extracellular NADH oxidase that functions in defense potentiation (Graham and Graham, 1999). The quick release of genistein during pathogen attack by the apoplastic isoflavone conjugate-hydrolyzing β-glucosidase may thus be critically important for several aspects of disease resistance.

Although previous non-specific β-glucosidases were reported to hydrolyze isoflavone glucosides in soybean (Matsuura and Obata, 1993; Matsuura et al., 1995), the currently purified soybean ICHG has higher specific activity towards isoflavone conjugates with substrate specificity constants 160-fold to 1000-fold over *p*-nitrophenyl β-glucoside (Table 3). The β-glucosidase B and C isolated from soybean cotyledons had a relatively low

reaction rates (79 and 38%, respectively) with isoflavone glucosides compared to *p*-nitrophenyl β-glucoside (100%) (Matsuura and Obata, 1993; Matsuura et al., 1995). The substrate binding affinities of ICGH towards isoflavone conjugates are comparable to the chickpea isoflavone-specific β-glucosidase and flavone-glucoside-cleaving β-glucosidase from *Charmomilla recutita* (in the submillimolar range), suggesting favored binding to their natural substrates (Hösel and Barz, 1975; Maier et al., 1993). However, chickpea isoflavone-specific β-glucosidase has apparently higher specificity constants toward formononetin and biochanin A glucosides ($\sim 10^6$ M^{-1} s^{-1}) than that of ICHG. Furthermore, Hg^+, Ag^+ and glucono-δ-lactone are all inhibitory to ICGH, consistent with other plant β-glucosidases, although ICGH is less sensitive to Hg^+ and glucono-δ-lactone compared to the chickpea enzyme. Although further characterization will be required to firmly determine this, the ICHG may hydrolyze isoflavone malonylglucosides directly. This may distinguish it from the chickpea β-glucosidase. Taken together, we conclude that the β-glucosidase purified from soybean roots is a novel β-glucosidase with high specificity toward isoflavone conjugates.

4. Experimental

4.1. Plant material

Soybean (*Glycine max* L.) cv. Williams was obtained from Dr. A. F. Schmitthnner (Department of Plant Pathology, The Ohio State University and the Ohio Agricultural Research and Development Center, Wooster, OH). Seedlings were grown as described previously (Graham, 1991a). Soybean roots from 7–9 day-old seedlings were washed with tap water to remove planting media and blotted with paper towels. The roots were frozen at −20 °C until used.

4.2. Isolation and purification of isoflavone conjugates

Isoflavone conjugate substrates were prepared as follows. Soybean cv. Williams seeds (50 g) were ground to a fine powder with a coffee grinder and the powder was extracted with 100 ml chloroform for 30 min with stirring. The slurry was filtered through Whatman No. 1 filter paper with suction and washed two more times with chloroform. The defatted soybean flour was extracted twice with 50 ml distilled water. The aqueous extract (100 ml) was clarified by low speed centrifugation (International Equipment Co., Needham, MA). The isoflavone conjugates of genistein and daidzein were partially purified using a C18 PrepSep column (Fisher Scientific, Springfield, NJ). The crude aqueous substrate preparation was passed through the column, and the column was washed successively with water and 1 ml of 10% acetonitrile (HPLC grade, Sigma Chemical Co., MO). The 10% eluent was discarded. Washing with 1 ml of 20% acetonitrile released bound daidzin, genistin, MGD and MGG. This provided a preparation of the isoflavone conjugates free of aglycones (which remained bound to the column and are eluted only at higher acetonitrile concentrations). For measurement of enzyme activity in initial protein fractionations, this isoflavone conjugate fraction was brought to dryness and dissolved in an appropriate amount of 100 mM phosphate–50 mM citrate buffer, pH 5.0 to make the concentrations of each isoflavone conjugate greater than 2.5 mM as judged by HPLC (Graham, 1991a). For final purification and characterization of the glucosidase, individual isoflavone conjugates were isolated by semi-preparative HPLC using a Merck Lichrosorb RP-18 10 mm C18 column (Alltech Associates, Deerfield, IL) with non-acidic water and acetonitrile as described by Graham (1991a).

4.3. Buffers

Buffer A, 200 mM sodium phosphate buffer, pH 6.0; buffer B, 200 mM sodium phosphate buffer, pH 6.0, containing 0.5 M NaCl; buffer C, 20 mM sodium phosphate buffer, pH 6.0, containing 3 mM DTT and 10% glycerol; buffer D, buffer C containing 1 M NaCl; buffer E, buffer C containing 5 mM sodium ascorbate; and buffer F, buffer D containing 5 mM sodium ascorbate.

4.4. Enzyme extraction and purification

Frozen roots were ground almost to a powder in a mortar and pestle pre-chilled to 0 °C and extracted three times with cold buffer A (1 ml buffer/ 1 g tissue). The combined extracts were centrifuged at 10,000×*g* for 10 min. The pellet was extracted with buffer B (1 ml buffer/ g tissue) overnight at 4 °C. The root residue was further extracted one more time with an equal volume of buffer B for another 3 h. The crude extracts were pooled and 1% (w/v) insoluble polyvinylpolypyrolidone (PVPP) was added to remove phenolics. This mixture was stirred for 3 h on ice. The extract was then filtered through four-layers of cheesecloth, and clarified by centrifugation at 10,000×*g* for 20 min. The resulting solution was slowly brought to 40–60% saturation with solid ammonium sulfate at 4 °C followed by gentle stirring overnight. The pellet was collected by centrifugation at 10,000×*g* for 20 min, and then dissolved in a minimum volume of buffer C. The protein solution was clarified by centrifugation and desalted using a Sephadex G-25 column (1.7×20 cm) equilibrated with buffer C. The desalted protein solution was applied to a DEAE-Sephadex A-50 column (2.8×35 cm) equilibrated with the same buffer system and washed with buffer C. The enzyme was found in the unbound fractions. Sodium

ascorbate was added to the protein solution to a final concentration of 5 mM, and the solution was then loaded onto a column of CM-Sephadex C-50 (2.8×19 cm), equilibrated with buffer E. The elution was carried out with a 400 ml linear gradient of buffer F in buffer E. Chromatographic fractions having ICHG activity were pooled and lyophilized. The concentrated enzyme was stored at −20 °C for further study.

4.5. Enzyme and protein assays

Protein concentration was determined by the Bradford (1976) method with BSA as standard. During purification and unless otherwise noted, ICHG activity was determined in 0.2 M phosphate–0.1 M citrate buffer, pH 5.0 at 40 °C in a total reaction volume of 10 μl containing 5 μl of isoflavone conjugate mixture. The reaction was stopped at 30 min by adding 90 μl of methanol and activity was measured by quantifying the release of aglycones by HPLC (Graham, 1991a). This enzyme assay allowed us to simultaneously examine the relative release of aglycones from both daidzein and genistein conjugates, and also allowed us to measure the possible esterase activities in fractions leading to the formation of daidzin and genistin from MGD and MGG, respectively. During the course of the fractionation, the glucosidic activities towards daidzein and genistein conjugates were always comparable and thus results are expressed in terms of total aglycone release. One unit of enzyme activity was defined as the amount of enzyme that produced 1 mmol of product per minute. Specific activity was expressed as enzyme units per mg protein. No apparent conversion of MGD or MGG to daidzin or genistin was observed in any extract or during any fractionation step; that is, we never observed an isoflavone-specific malonylesterase activity as reported in chickpea (Hösel and Barz, 1975).

In the study of substrate specificity, the concentration of substrate was 2 mM unless otherwise noted. Daidzein 7-*O*-glucoside, daidzein 7-*O*-glucoside-6″-*O*-malate, genistein 7-*O*-glucoside, and genistein 7-*O*-glucoside-6″-*O*-malate were assayed at 0.2, 0.18, 0.79 and 1.15 mM, respectively. The appropriate concentration of the ICHG used for each substrate was determined graphically from the linear portions of initial reaction velocity. All substrates were dissolved in 0.2 M phosphate–0.1 M citrate buffer, pH 5.0, except that *p*-nitrophenyl α-glucoside, 4-methylumbelliferyl β-glucoside, and genistein 7-*O*-glucoside were dissolved in ethylene glycol monoethyl ether at 50 mM and then diluted to final concentration in the same buffer.

Depending on the substrate, activity was measured either spectrophotometrically or by HPLC. For those substrates releasing an aglycone with known molar coefficient (ϵ), the assay solution containing the enzyme (10 μl) and substrate (90 μl) in 0.2 M phosphate–0.1 M citrate buffer, pH 5.0, was incubated at 40 °C for various time courses. The reaction was stopped by alkalization of the assay mixture with 100 μl of sodium carbonate, and the mix was then diluted to 1 ml with distilled water for measurement of absorbance. For quantitative calculations, the following wavelengths and ε values (mM^{-1} cm^{-1}) were used: *p*-nitrophenol, 400 nm, $\varepsilon = 19.3$; *o*-nitrophenol, 420 nm, $\varepsilon = 4.55$; 4-methylumbelliferone, 360 nm, $\varepsilon = 18.25$; and salicyl alcohol, 295 nm, $\varepsilon = 3.3$. For the substrates, esculin and arbutin, without known ε values, the following method was developed. The UV spectrum of esculin showed a peak at 374 nm and the absorbance intensity decreased over time with enzymatic activity. The activity assay was conducted as described above, and the absorbance change was converted to concentration by a linear standard curve made from various concentrations of esculin in 100 μl Na_2CO_3 and 900 μl water. The enzyme activity against arbutin was determined similarly at 267 nm.

Activity against several natural substrates was assayed by the HPLC method for isoflavone conjugates as described above for enzyme purification, except that the total reaction volume was 15 μl and the reaction was stopped at various times by adding 85 μl of methanol. The substrates assayed by HPLC included the four isoflavone conjugates, two flavonol glycosides (rutin and isoquercitrin), phloridzin (a chalcone glucoside), and amygdalin (a cyanogenic di-glucoside). Commercial almond emulsin β-glucosidase, a classic non-specific β-glucosidase, was also examined to compare substrate specificity. All K_m and V_{max} values were calculated based on Lineweaver-Burk plots, and the k_{cat} value is equal to V_{max} divided by the total enzyme concentration (in molarity) which was calculated using M_r of 165 kDa for ICHG and 135 kDa for almond emulsin β-glucosidase. The kinetic parameters reported are the mean values of two or three independent determinations.

The temperature optimum of the ICHG was resolved by incubating the enzyme solution with isoflavone conjugates over a temperature range from 0 to 90 °C for 30 min. Enzyme activity was monitored by the standard HPLC method. To determine the pH optimum, 4-methylumbelliferyl β-glucoside was used in various buffers varying in pH values from 2.6 to 10.6. Enzyme activity was determined spectrophotometrically as described above.

4.6. SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed as described by Laemmli (1970) using a Mini-Protein II electrophoresis system (Bio-Rad). Analyses were carried out under non-reducing or reducing (mercaptoethanol) conditions as specified. The molecular markers used were bovine serum albumin (66 kD), ovalbumin (45 kD), and carbonic

anhydrase (29 kD) all from Sigma Chemical Co. Protein gels were stained with silver stain (Bio-Rad).

4.7. Inhibition assays

Several inorganic and organic compounds and solvents were examined for their inhibitory effects. The reaction mixture was in 0.2 M phosphate–0.1 M citrate buffer, pH 5.0 using a total reaction volume of 15 μl incubated at 40 °C for 30 min; the reaction was stopped by adding 85 μl of methanol. The enzyme activity was measured by HPLC and designated as 100% in the absence of added chemicals. The activity reported is the average from two independent measurements. A series of concentrations of Ag^+, β-mercaptoethanol and glucono-δ-lactone, respectively, were further inspected to determine the concentration for 50% inhibition (IC_{50}). For prevention of inhibition of activity by silver ions, 250 mM of β-mercaptoethanol was incubated with 5 μl of isoflavone-conjugate substrate and 5 μl of various concentrations of Ag^+ for 20 min before the enzyme was introduced. IC_{50} values are obtained from the least square fit to the Langmuir isotherm equation, $v_i/v_0 = 1/(1 + [I]/IC_{50})$, using Igor Pro software (WaveMetrics, Inc., OR), where v_i is the velocity in the presence of inhibitor at concentration [I] and v_0 is the velocity in the absence of inhibitor.

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