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Properties of β-thioglucoside hydrolases (TGG1 and TGG2) from leaves of *Arabidopsis thaliana*

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ABSTRACT

Myrosinases (β-thioglucoside glucohydrolase, TGG; EC 3.2.1.147) catalyze the hydrolysis of glucosinolates, a structurally distinct group of nitrogen- and sulfur-containing secondary metabolites, to give a chemically unstable intermediate, glucose and sulfate. This catalysis initiates a chemical defense in crucifer plants as a response to the tissue-damaging activities of herbivores and pathogens. To characterize the individual and collective biochemical properties of the myrosinase enzymes found in the aerial tissues of *Arabidopsis thaliana*, we purified TGG1 and TGG2, which share 73% amino acid identity, individually from T-DNA insertion lines of Arabidopsis using lectin affinity and anion exchange chromatography. Electrophoresis under denaturing conditions and the mobility of nondenatured TGG1 and TGG2 protein on gel filtration chromatography indicated that the native proteins exist as dimers of 150 and 126 kDa, respectively.

Despite their relatively similar kinetic parameters, both enzymes had distinct physicochemical properties such as extractability in low ionic strength buffer and electrophoretic mobility following deglycosylation treatment. Deglycosylation under nondenaturing conditions had limited effects on TGG1 and no effect on TGG2 activity. Both enzymes functioned across a broad range of temperatures (up to 60 °C) and pH values (5–10). These results demonstrate that myrosinases have the ability to function in environments like the digestive tract of insect herbivores that are significantly different from the environment in a damaged plant.

1. Introduction

Myrosinases (thioglucoside glucohydrolase, EC 3.2.3.147) and their glucosinolate substrates are the principal non-host chemical defense of crucifer plants against tissue-damaging pests. Myrosinases catalyze the cleavage of the *S*-glycosidic bond common to all glucosinolate structures using ascorbate as a cofactor and a reaction mechanism that retains the anomeric configuration at the cleavage site of the glucose moiety to produce an unstable intermediate that rearranges to form various cytotoxic compounds that have potent antiherbivore activities [1,2].

Additional enzymes that catalyze the breakdown of glucosinolates have been characterized including an enzyme from the aphid *Brevicoryne brassicae*, which sequesters glucosinolates extracted from its crucifer hosts [3,4]. And recently, the *PEN2* gene (At2g44490, *BGLU26*) of *Arabidopsis thaliana* has been shown to encode a myrosinase-like activity which is limited to indolic glucosinolate substrates [5]. Together these three classes of enzymes with thioglucoside glucohydrolase activity belong to a large family of β-glucosidases [6,7] which share certain structural features [1,8].

Myrosinases have been purified and characterized from various species of the Brassicaceae [9–16]. Typically, myrosinases are encoded by a multigene family organized by amino acid sequence homology into two groups of glycoside hydrolases, GH1 and GH2. Plant GH1 myrosinases are dimeric glycoproteins with 9–23% carbohydrate content, a zinc ion that stabilizes protein dimerization and a native molecular mass range of 125–150 kDa [17]. Based on tertiary structural determinations of 17 myrosinases of the GH1

Abbreviations: 4-MUG, 4-methylumbelliferyl-β-D-glucoside; Endo H, endo-β-*N*-acetylglucosaminidase; Onpg, *o*-nitrophenyl-β-D-glucopyranoside; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; pNPG, *p*-nitrophenyl-β-D-glucopyranoside; PNGase, peptide *N* glycosidase F; PGO, peroxidase-glucose oxidase coupled reaction; TGG, β-thioglucoside glucohydrolase.

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family, all enzymes have essentially the same $(\beta/\alpha)_8$ barrel fold structure even though they share only 17–44% sequence identity. The active site of these enzymes has a hydrophobic pocket for binding the variable but generally hydrophobic side chain of glucosinolates and includes specific amino acids that participate in catalysis or interact with the ascorbate cofactor or the glucose or sulfate moieties of glucosinolates [18].

The myrosinase gene family in Arabidopsis consists of six members all with amino acid sequence homologies to other members of the GH1 family [6]. Four of the six genes encode active myrosinases; two genes, *TGG1* (AGI code no. At5g26000) and *TGG2* (At5g25980), are expressed in aerial tissues and have 73% sequence identity at the amino acid level while *TGG4* (At1g47600) and *TGG5* (At1g51470) have 98% sequence identity and are expressed in subterranean tissues [19]. The two differentially expressed groups share 50% amino acid identity. The remaining members *TGG3* (At5g48375) and *TGG6* (At1g51490) are considered pseudogenes because of disrupted open reading frames, although these genes are transcribed and transcripts have been shown to accumulate in pollen [20,21].

We describe here the purification and characterization of TGG1 and TGG2 proteins in order to identify the biochemical properties of individual plant myrosinases and their collective properties in aerial plant tissue. The proteins were isolated from Arabidopsis mutants that accumulate only one or the other myrosinase enzymes in aerial tissues [22]. The identity of the purified protein was confirmed by size and detection with site-specific antisera. Purified TGG1 and TGG2 were compared for their physicochemical and catalytic properties. We also studied the deglycosylation of nondenatured TGG1 and TGG2 and its effect on enzyme activity and protein stability. Our results suggest a broader role for plant myrosinase activity in the chemical defense response than has been traditionally ascribed.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of wild type Arabidopsis Col-0, T-DNA insertion lines SALK_069615 (Stock no. CS25099; *tgg1*) and SALK_038730 (Stock no. CS25054; *tgg2*), were obtained from the Arabidopsis Biological Resources Center (ABRC, www.arabidopsis.org/abrc). The lines have been characterized for insertion site and lack of *TGG* transcript [22]. Plants were grown in soilless potting medium in a controlled environmental chamber with a 16-h photoperiod (200 μmol m^{-2} s^{-1}), 50% relative humidity at 22 °C (day) and 20 °C (night). For T-DNA mutant screening, surface-sterilized seeds were germinated on 0.5× Murashige and Skoog solid medium containing 30 μg ml^{-1} kanamycin. Homozygous T-DNA insertion lines of *tgg1* and *tgg2* were identified by PCR using genomic DNA as templates with gene-specific primers (Table 1).

2.2. Bioinformatic analyses

The amino acid sequences for various β-glucosidases were aligned using MegAlign (Clustal W with default parameters, DNASTAR Lasergene Ver. 8). *N*-glycosylation sites in the amino acid sequences were predicted by NetNGlyc Ver. 1.0 and *O*-glycosylation sites by NetOGlyc Ver. 3.1 [23] at the Center for Biological Sequence Analysis website (http://www.cbs.dtu.dk/services).

2.3. Generation of antibodies

Immunogenic peptides representing unique sequences from TGG1 and TGG2 (Table 1) were chemically synthesized (GenScript Corporation, Scotch Plains, NJ). After reduction and purification, the peptides were conjugated to bovine serum albumin (BSA), purified and desalted as described (Imject Maleimide Activated BSA Kit, Pierce, Rockford, IL). Immunospecific polyclonal antibodies against the purified BSA-conjugates were raised in rabbits as described previously [24].

2.4. Extraction and purification of TGG1 and TGG2 from Arabidopsis plants

For TGG1 extraction, 100 g of 4-week-old rosette leaves of *tgg2* mutant plants were homogenized in 100 ml of 20 mM sodium phosphate buffer, pH 7.0, containing 10% glycerol, 2 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (PMSF). TGG2 was extracted under similar conditions except that *tgg1* mutant plants were used and the extraction buffer contained 0.5 M NaCl. The homogenates were incubated on ice for 2 h and centrifuged at 12,000 × *g* at 4 °C for 20 min. The pellets were reextracted under the same conditions as described above. The combined supernatant of the two extractions was filtered through a 0.45 μm membrane (Pall Corporation, NY) and loaded onto a Concanavalin A-Sepharose 4B column (1.5 cm × 2.5 cm; Amersham Biosciences, NJ) equilibrated with 20 mM Tris–HCl, 0.5 M NaCl, pH 7.4, and washed with 100 ml of the same buffer. Protein was eluted with a 150 ml linear gradient of 0–300 mM methyl-α-D-mannopyranoside (Sigma–Aldrich, St. Louis, MO) in 20 mM Tris–HCl, pH 7.4, at a constant flow rate of 2 ml min^{-1}. Myrosinase-containing fractions were pooled and loaded onto an Accell QMA column (1.5 cm × 2.5 cm; Waters, Milford, MA), equilibrated with 10 mM potassium phosphate, pH 7.0. The column was washed with 100 ml of the same buffer and protein eluted with a 150-ml linear gradient of 0–300 mM NaCl in the phosphate buffer, at a flow rate of 2 ml min^{-1}. The peak fractions were collected. Protein determinations were made with a Bradford protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

2.5. Myrosinase enzyme assay

Myrosinase catalytic activity was assayed using sinigrin as a glucosinolate substrate if not stated otherwise. Sinigrin was varied from 0 to 10 mM in 50 mM citrate–100 mM phosphate buffer, pH 5.8, in the absence or presence of 0.63 mM L-ascorbic acid and 0.13 μg of purified protein and incubated at 25 °C for 30 min. The activation of myrosinase by ascorbic acid was measured similarly with 1 mM sinigrin and the assay buffer adjusted to a final concentration of 0.04–1.25 mM ascorbic acid. The enzyme reaction was stopped by heating the mix at 95 °C for 10 min and the released glucose measured by the peroxidase-glucose oxidase (PGO) coupled reaction assay [6]. The data were analyzed with Enzymekinetics V1.5 (Trinity Software, Campton, NH) as described previously [25]. The apparent k_{cat} values were calculated based on one active site per unmodified monomeric polypeptide with molecular weights of 61.1 and 62.7 kDa for TGG1 and TGG2, respectively.

2.6. Modification of pH and temperature parameters of the myrosinase assay

To measure myrosinase activity in a range of pH values, TGG1 and TGG2 activity was determined in the presence of 20 mM buffers including citrate (pH 3.0–5.0), phosphate (pH 6.0–8.0), Tris–HCl (pH 8.0–9.0), and carbonate (pH 10.0–11.0) buffers. To measure pH stability, TGG1 and TGG2 were incubated in buffers of different pH values at 25 °C for 30 min. The solutions were diluted 4-fold with 200 mM citrate phosphate buffer, pH 5.8, and assayed for myrosinase activity. TGG1 and TGG2 were incubated at temperatures from 10 °C to 95 °C for 30 min in the presence of 0.2 mM