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# Immunopurification and Immunocharacterization of the Glucosinolate Biosynthetic Enzyme Thiohydroximate *S*-Glucosyltransferase

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**Preparing homogeneous UDP-glucose:thiohydroximate *S*-glucosyltransferase (S-GT), the penultimate biosynthetic enzyme of glucosinolates, by standard chromatographic methods has yielded too little protein for adequate purity evaluation, identity verification, and structural analysis. The low yields were apparently due to low abundance in source tissues, aggravated by enzyme instability. Here we describe an immunological method for purification of workable quantities from florets of *Brassica oleracea* ssp. *botrytis* (cauliflower). Florets that had undergone browning due to exposure to sunlight contained higher S-GT activities than are normally found in *Brassica* tissues. S-GT was adsorbed from crude tissue extracts onto an agarose-monoclonal antibody complex. Elution from the complex required harsh alkaline conditions (pH 11.5), giving extremely variable activity recoveries (maximum 20%). The eluate contained two proteins that could be separated readily by preparative polyacrylamide gel electrophoresis or anion-exchange chromatography. The overall S-GT protein recovery was estimated at less than 200 μg/kg of cauliflower tissue. Molecular weight determinations with homogeneous cauliflower S-GT gave relative molecular weight ($M_r$) values of 55,500 as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 57,600 by gel chromatography; isoenzymes with isoelectric point values of 4.80 and 4.95 were identified. A polyclonal antibody raised against denatured enzyme showed broad cross-reactivity in immunoblots with S-GT from a number of *Brassica* species and other crucifers. The monoclonal antibody that was used in the immunopurification was much more specific; it exclusively precipitated S-GT isoenzymes that had their genomic origin in the primary diploids *B. oleracea* and *Brassica campestris*. Thus, all of the S-GT was precipitated from the amphidiploid *Brassica napus*, which is a hybrid of *B. oleracea* and *B. campestris*. About half of the S-GT was precipitated from the amphidiploids *Brassica carinata* and *Brassica juncea*, which have *B. oleracea* and *B. campestris* as one of their parents, respectively. It was shown that the S-GT isoenzymes of *B. juncea* with $M_r$ 55,500 and about 57,000 originate from the parents *B. campestris* and *B. nigra*, respectively.**

GS (sulfated *S*-glucosyl thiohydroximates) constitute a large group of secondary metabolites occurring in several plant families, including all species of the Cruciferae. Their presence in oilseed *Brassica* crops (*Brassica napus* and *Brassica campestris*) has been of great nutritional and therefore economic concern, since the meal fraction is directed to animal feed markets as a protein source. GS have antinutritional properties and cause acute and chronic diseases, particularly monogastrics, in domestic animals (Campbell, 1987; Bell et al., 1991). Plant breeding has already reduced the overall levels significantly (approximately 10-fold), but efforts to reach near-zero levels are continuing (Roebbelen, 1981; Love et al., 1990) (variety development priorities for 1993, formulated by the Canola Council of Canada, Winnipeg). Our ultimate goal is to generate novel germplasm with down-regulated genes for GS biosynthesis through genetic engineering.

Biosynthesis of GS involves the oxidation of the amino group of amino acids (in *Brassica* primarily Trp and homologs of Met), followed by oxidation/decarboxylation to aldoximes (Bennett et al., 1993; Poulton and Moller, 1993). Detoxification, translocation, and vacuolar storage (Hoesel, 1981; GrootWassink et al., 1987) are subsequently facilitated by conjugation reactions that successively introduce a sulfur, a glucosyl, and a sulfate moiety (Jain et al., 1990b). Plant feeding studies have shown that enzymes catalyzing these conjugation reactions are very nonspecific for the side chain, indicating that different GS in a plant are formed by the same enzymes (GrootWassink et al., 1987, 1990). Modulating the expression of their encoding genes would thus affect the formation of all GS.

Homogeneous enzyme protein was sought for primary structure determination. Much effort has been expended in our laboratory to purify the glucosylation and sulfation enzymes from *B. napus* seedlings and *Brassica juncea* tissue culture via conventional chromatographic techniques (Jain et al., 1990a, 1990b; Reed et al., 1993). Others have attempted purification from cress (*Lepidium sativum*) seedlings (Glendening and Poulton, 1988). However, low abundance, instability, co-purification of contaminant proteins with similar physico-chemical properties, and inconsistent recoveries have hindered the routine preparation of homogeneous enzymes. Here we report a short and reliable method, involving immunoaffinity precipitation, for the complete purification of the glucosylation enzyme S-GT (EC 2.4.1.–). The structural relatedness of this enzyme from different *Brassica* species and other Cruciferae species was studied with monoclonal and polyclonal antibodies.

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Abbreviations: FPLC, fast-performance liquid chromatography; GS, glucosinolate; K, thousand; 2-ME, 2-mercaptoethanol; pI, isoelectric point; S-GT (or GT), UDP-Glc:thiohydroximate *S*-glucosyltransferase.

## MATERIALS AND METHODS

### Buffers

Extraction buffer: 0.2 M Hepes (pH 7.5) containing 5 M EDTA and 0.1% 2-ME (v/v). Universal buffer: 20 mM Tris-HCl (pH 7.5) containing 30% glycerol (w/v) and 0.1% 2-ME. PBS: 0.2 g/L $KH_2PO_4$ and 2.16 g/L $Na_2HPO_4$ (pH 7.5) plus 8 g/L NaCl, 0.2 g/L KCl, 0.1 g/L $MgCl_2 \cdot 6\ H_2O$, and 0.13 g/L $CaCl_2 \cdot 2\ H_2O$. SDS-PAGE sample buffer: 120 mM Tris-HCl (pH 6.8) containing 4% SDS (w/v), 5% 2-ME (v/v), 20% glycerol (w/v), and 0.02% bromphenol blue (w/v).

### Enzyme Source

S-GT was extracted from the florets (head) of locally grown open type *Brassica oleracea* ssp. *botrytis* cv Snowball (cauliflower). Discolored plants with light-brown to dark-brown appearance ("sunburnt") were chosen for their higher S-GT activity. Tissues were homogenized for 2 min with equal volumes of extraction buffer (w/v) in a Waring blender and subsequently passed through a household juice extractor. Residual liquid was squeezed from the pulp through fine nylon mesh. Extracts were stored frozen at −20°C without loss of activity for 1 year.

### Enzyme Isolation

During the early part of this study S-GT was routinely precipitated from cauliflower extracts with 40 to 70% saturated ammonium sulfate, then redissolved in and dialyzed (25K mol wt cutoff) against universal buffer. Later, the ammonium sulfate step was omitted and crude extracts were dialyzed directly against universal buffer containing 5 mM EDTA. After centrifugation (30 min, 15,000*g*) and microfiltration (0.8 μm), batches of ammonium sulfate-precipitated enzyme or dialyzed extracts equivalent to 400 g of original tissue were subjected to FPLC anion-exchange chromatography (Mono Q HR16/10, Pharmacia). S-GT was eluted with a gradient of NaCl (0–500 mM) in universal buffer. Pooled active fractions (at about 0.20 M NaCl) were concentrated and washed with universal buffer by ultrafiltration using a stirred cell (10K mol wt cutoff, Amicon). The concentrate was then subjected to preparative continuous native PAGE (pH 8.8, 0.1% 2-ME in buffer and gel) using slab gels (15%, 6 cm long; Mini-Protean cell, Bio-Rad) (Davis, 1964; Ornstein, 1964). A 3-mm-wide strip was cut vertically from the center of the gels and subsequently sliced horizontally into strips 1 mm wide. S-GT activity was located on the gel by incubating the 1-mm strips directly in the regular assay mixture. The active region was excised from the gel and the enzyme was recovered by electroelution (Schleicher & Schuell Elutrap) using 15 g/L Tris-72 g/L Gly buffer (pH 8.3) containing 20% glycerol and 0.1% 2-ME. The eluate was concentrated by ultrafiltration (Centricon 10, Amicon).

Enzyme purity was evaluated by discontinuous SDS-PAGE according to Laemmli (1970) using slab gels (15%, 6 cm long) and Coomassie blue R-250 staining. Samples were denatured and reduced by heating in equal volumes of SDS-PAGE sample buffer. Progress of native PAGE and SDS-PAGE runs was monitored visually with prestained protein markers, including phosphorylase B (106K), BSA (80K), ovalbumin (49.5K), carbonic anhydrase (32.5K), soybean trypsin inhibitor (27.5K), and lysozyme (18.5K) (Bio-Rad). Standards for SDS-PAGE mol wt determination were the same proteins but unstained, i.e. phosphorylase B ($M_r$ 97.4K), BSA ($M_r$ 66.2K), ovalbumin ($M_r$ 45K), carbonic anhydrase ($M_r$ 31K), trypsin inhibitor ($M_r$ 21.5K), and lysozyme ($M_r$ 14.4K) (Bio-Rad). Standards for mol wt determination by FPLC gel filtration (Superose 12, Pharmacia) were alcohol dehydrogenase ($M_r$ 150K), BSA (66.2K), carbonic anhydrase ($M_r$ 29K), and Cyt *c* ($M_r$ 12.4K) (Sigma). Analytical thin-layer polyacrylamide IEF of deionized enzyme preparations was carried out in a horizontal apparatus using a urea-free ampholyte system and Coomassie blue R-150 stain (method adopted from Bio-Rad's Bio-Phoresis Horizontal Electrophoresis Cell instruction manual). Standards used were Glc oxidase (pI 4.50), β-lactoglobulin B (pI 5.10), bovine carbonic anhydrase (pI 6.00), human carbonic anhydrase (pI 6.50), equine myoglobin (pI 7.00), human hemoglobin A and C (pI 7.10 and 7.50), lentil lectin (pI 7.8, 8.0, 8.2), and Cyt *c* (pI 9.60).

### Enzyme Assay

The S-GT assay was based on the incorporation of [$^{14}$C]Glc from UDP-Glc into phenylacetothiohydroximate as described by Reed et al. (1993). The reaction mixture (total volume 100 μL) consisted of 80 μL of 50 mM Mes buffer (pH 6.0) containing 1.0 mM UDP-[*U*-$^{14}$C]Glc (0.05 μCi; NEN), 1.0 mM phenylacetothiohydroximate, 5 mM $MgSO_4$, and 0.1% 2-ME plus 20 μL of enzyme solution. Incubation at 30°C varied from 5 to 30 min to remain below 30% substrate utilization, i.e. assay linearity range. The reaction was stopped by heating at 90°C, and after cooling the radioactive end product was extracted by vigorous shaking with 0.5 mL of ethyl acetate. The organic solvent phase was clarified by centrifugation, 0.4 mL was added to 2.5 mL of high flash-point liquid scintillation cocktail (Opt-fluor, Packard Instrument Co., Downers Grove, IL), and the radioactivity was measured in a scintillation counter (LKB Rackbeta 1219). Protein was determined according to Bradford (1976) using BSA as standard.

### Monoclonal Antibody Production and Immunoaffinity Purification

All methods for antibody production, immunoaffinity separation, and immunoblotting were adopted from the laboratory manual of Harlow and Lane (1988). Mice were immunized with partially purified (580-fold) S-GT from *B. napus* seedlings (Reed et al., 1993). Mouse splenocytes were fused with myeloma cells and the resulting hybridoma cell lines were screened for binding of S-GT activity as follows (Dr. L. Qualtiere, L. McDonald, and T. Archer, Department of Microbiology, University of Saskatchewan, Saskatoon, personal communication). Hybridoma tissue culture supernatant (100 μL) was mixed with agarose-goat anti-mouse IgG antibody (10 μL; Organon Teknika Corp., West Chester, PA) in a microcentrifuge tube, shaken for 3 h at 4°C, centrifuged (15 s, 12,000*g*), and the agarose pellet was washed once with 1 mL of PBS buffer. Then, an S-GT solution (50 μL; same as used in immunization) was added to the agarose pellet,

shaken for 1 h at 4°C, and centrifuged. The agarose pellet was washed twice with 1 mL of PBS buffer. Finally, the S-GT activity that was adsorbed to the agarose-antibody complex was assayed by resuspending the agarose pellet in 80 μL of enzyme reaction mixture and incubated with shaking for 1 h at 30°C (see above for assay details). Out of almost 800 cell lines, one was found to produce an antibody that precipitated S-GT activity.

The selected hybridoma cell line was injected into the peritoneum of mice and ascitic fluids containing large amounts of monoclonal antibody were collected. For preparation of an immunoaffinity matrix, the antibodies from ascitic fluids (2 mL, 2.5 mg/mL protein) were first adsorbed onto agarose-goat anti-mouse IgG antibody complex (2 mL) by incubating the mixture overnight at 4°C with gentle shaking. The agarose was then recovered by centrifugation (2 min, 800*g*) and washed three times in 15 mL of PBS buffer. For covalent coupling of the sandwich antibody complex, the agarose was resuspended in 0.2 M sodium borate buffer (pH 9.0) and solid dimethylpimelimidate (20 mM) was added. After 1 h of incubation at room temperature with gentle shaking, the reaction was stopped by centrifugation and resuspension of the agarose pellet in 12 mL of 0.2 M ethanolamine (pH 8.0). Noncovalently bound proteins were removed by successive washings of the agarose with 12 mL of PBS, 0.1 M triethylamine (pH 11.5), PBS, deionized water, and 1 M NaCl in PBS and PBS.

For immunoaffinity purification of S-GT, crude cauliflower extracts were dialyzed (25K mol wt cutoff) overnight against universal buffer containing 0.1 M NaCl and 5 mM EDTA. Agarose-monoclonal antibody complex (1.5–2 mL) was added to 250 to 350 mL of dialyzed extract and incubated overnight at 4°C with gentle shaking. The agarose was recovered by centrifugation (2 min, 800*g*) and washed successively with 12 mL of PBS, deionized water, and 0.1 M NaCl in PBS and PBS. The adsorbed proteins, including S-GT, were eluted by resuspension of the agarose-antibody enzyme complex in 12 mL of ice-cold 0.1 M triethylamine (pH 11.5) followed immediately by centrifugation and neutralization of the supernatant in an equal volume of cold 0.14 M Tris-HCl (pH 7.5) containing 30% glycerol and 0.1% 2-ME. The eluate was concentrated to about 0.2 mL and washed with 5 mL of universal buffer by ultrafiltration in a stirred cell (10K mol wt cutoff, Amicon).

### Polyclonal Antibody Production and Immunoblotting

Immunopurified proteins from dialyzed crude cauliflower extracts were separated by SDS-PAGE and Coomassie stained. The S-GT protein band (approximately 55K) was excised, washed overnight with 30 mL of deionized water, and macerated in complete or incomplete Freund's adjuvant for rabbit immunization. Complete Freund's adjuvant was used for the first injection only. Intramuscular injections (25–50 μg enzyme protein/dose) were given every 3–5 weeks for a total of five injections. In immunoblot analysis, antiserum was used either directly or after isolation of the IgG fraction by 50% saturated ammonium sulfate precipitation and Sephadex G-200 chromatography. A 1:1000 dilution of antiserum gave a visible band with 1 μg of pure S-GT in 10 s of immunoblot staining.

Immunoblots were prepared by electrophoretic transfer of proteins from PAGE gels to nitrocellulose membranes using a semidry apparatus (Transblot SD, Bio-Rad). The main conditions for immunoblot probing and staining were as follows (Harlow and Lane, 1988). (a) Blocking of nitrocellulose with skim milk powder (50 g/L) in PBS buffer for 2 to 3 h at room temperature. (b) Incubating with rabbit S-GT antiserum at 1:1000 dilution at 4°C overnight. (c) Incubating with the secondary antibody goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) at room temperature for 2 to 3 h. (d) Staining with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

### Specificity of Monoclonal and Polyclonal Antibodies

Cross-reactivity of antibodies was determined with S-GT and other proteins from several crucifers. Seedlings were grown to the three-leaf stage in a growth chamber (25°C, 16 h light/8 h dark) or plant material was collected from local gardens and fields. Tissues (5–50 g) were homogenized (Omni mixer, Sorvall, Inc.) in 2 volumes of cold extraction buffer and centrifuged (20 min, 15,000*g*). For immunoprecipitation with monoclonal antibody, 12-mL aliquots of crude extracts that had been clarified by centrifugation and microfiltration were added directly to a 10-fold excess (approximately 1.5 mL) of agarose-monoclonal antibody complex. The mixture was incubated at 4°C with gentle shaking for 3 h. After centrifugation (2 min, 800*g*), S-GT was assayed in the supernatant. Control assays omitting the thiohydroximate from the reaction mixture were done to correct for glucosylation of endogenous substrates present in the crude extracts.

For immunoblotting, extracts of plant tissues were precipitated with ammonium sulfate (30–70% saturation) and chromatographed on Superose 12 gel (Pharmacia; column 16 mm wide/50 cm long) using universal buffer plus 0.1 M NaCl as eluant. S-GT active fractions were combined, concentrated by ultrafiltration, and subjected to SDS-PAGE (10%) and immunoblot analysis. The gel-filtration step was necessary to remove an abundant protein whose subunit comigrated with S-GT in SDS-PAGE and blocked detection of S-GT in immunoblots.

## RESULTS AND DISCUSSION

### Enzyme Sources

Plant tissues we used previously to isolate S-GT were cell cultures of *B. juncea* and dark-grown seedlings of *B. napus* (Jain et al., 1990a, 1990b; Reed et al., 1993). The extremely low levels of S-GT in these tissues required the processing of large quantities, prompting us to seek a more readily available alternative among vegetable *Brassica* species. Comparable enzyme activities were present in white cauliflower of the self-blanching type, hybrid cultivars commonly available. However, higher activities were found in locally grown cauliflower of the open type that had been allowed to turn brown in the field due to exposure to sunlight (Table I). This condition, referred to as sunburn, varied greatly among the plants and seemed to reflect the variation in S-GT activity

**Table I.** *Sources of thiohydroximate S-glucosyltransferase*

| Crude Extract | Glucosyltransferase Activity | |
|---|---|---|
| | *units mg$^{-1}$ protein* | *units kg$^{-1}$ tissue* |
| *B. napus* seedlings | 0.75 | 9,250 |
| *B. juncea* cell culture | 2.2 | 11,800 |
| *B. oleracea* ssp. *botrytis* (cauliflower) florets | | |
| Self-blanching type (white) | 3.7 | 16,340 |
| Open type (brown) | 44 | 317,800 |

[a] One unit of enzyme produced 1 nmol of benzyldesulfoglucosinolate min$^{-1}$.

(up to 10-fold). Discolored plants were chosen for enzyme extraction.

Analysis by high-pressure liquid chromatography (GrootWassink et al., 1990) indicated that the GS content, in particular that of 1-methoxy-3-indolylmethyl GS (Carlson et al., 1987), was extremely high in cauliflower florets that had undergone extensive browning (data not shown). This suggests that environmental factors such as intensity and extent of sunlight exposure may play a role in the activity of the biosynthetic pathway in cauliflower. Accumulation of GS in vegetative tissues of *B. napus* is reported to depend on the nutritional state and stress conditions such as mechanical damage, insect feeding, and fungal infection (Milford et al., 1989; Doughty et al., 1991; Koritsas et al., 1991; Bodnaryk, 1992). Yearly monitoring of the rapeseed crop (*B. napus* and *B. campestris*) in Canada has revealed that above-average levels of GS occur in seed produced during a growing season marked by high temperatures and drought (Canadian Grain Commission). Similar seasonal variations have been observed in Great Britain (Morgan and Cooke, 1989; Milford and Evans, 1991).

The apparent correlation between GS and S-GT levels in cauliflower was not evident when high-GS and low-GS rapeseed cultivars were compared. Their S-GT activities appeared to be indistinguishable in young leaves of plants grown side by side in a growth chamber.

## Enzyme Specificity and Occurrence

Over the course of this purification study, all enzyme preparations other than immunopurified S-GT showed varying degrees of *O*-glucosylation activity toward the flavonols kaempferol and quercetin. The ability to separate S-GT from this widely occurring flavonol glucosyltransferase activity (Sun and Hrazdina, 1991) provides further evidence that S-GT is specific for thiohydroximates. The enzyme does not act on oxygen analogs of hydroximates (Reed et al., 1993). However, S-GT lacks specificity for the side group of thiohydroximate, as was shown earlier by feeding experiments with a wide variety of natural and unnatural glucosinolate precursors (GrootWassink et al., 1987), and recently by demonstrating in vitro activity toward aliphatic and indole thiohydroximates (Reed et al., 1993).

To substantiate the role of S-GT in glucosinolate metabolism, we examined its occurrence in GS-producing and non-producing plants. The presence of S-GT activity was confirmed in the organs of several crucifers, i.e. in the roots, stems, leaves, and pods of *Brassica* species, *Sinapis alba*, *Thlaspi arvense* (stinkweed), *Descurania sophia* (flixweed), and *Arabidopsis thaliana*. Interestingly, S-GT and GS were absent from the rosette leaves of *Capsella bursa-pastoris* (shepherd's purse), a cruciferous weed that nevertheless contains S-GT and GS in the roots, stems, raceme leaves, and pods (GrootWassink et al., 1991). The GS-degrading enzyme myrosinase is also absent from the rosette leaves, suggesting that, in shepherd's purse at least, the total GS metabolic complex is developmentally controlled. Finally, S-GT activity was not detected in GS-free plants, including carrot, soybean, and alfalfa.

## Chromatographic and Electrophoretic Fractionation

To prepare a small quantity (10–20 $\mu$g) of purified S-GT from 2 kg of *B. napus* seedling tissue by chromatographic techniques proved to be a long and arduous task (Reed et al., 1993). Purification was hampered severely by low abundance in the source material, compounded by inconsistent and poor activity recoveries due to instability and physical losses in each step.

In the present work, the instability problem was alleviated somewhat by including 30% glycerol in all buffers other than the extraction buffer. An accelerated inactivation test at 30°C showed that glycerol increased the half-life of ammonium sulfate-precipitated S-GT activity from 4.7 to 16.3 h. Glycerol also allowed freezing and thawing of enzyme solutions without loss of activity.

With cauliflower as S-GT source of higher activity, we tried to reduce the number of purification steps and improve the yields. Two well-established steps were initially maintained, ammonium sulfate fractionation and anion-exchange chromatography (Jain et al., 1990a). However, the effectiveness of the salting-out step became questionable, particularly with high-activity extracts. Major losses (up to 60%) were incurred with only a 2-fold increase in specific activity. Thus, this step was omitted later and tissue extracts were dialyzed directly for anion-exchange chromatography. After clarification, varying amounts of activity (up to 40%) were still lost. Adding protease inhibitors (leupeptin, PMSF, benzaminidine, $\epsilon$-amino-caproic acid), phenoloxidase inhibitor (diethyldithiocarbamate), and a phenol adsorbent (polyvinylpolypyrrolidone) to the extraction and dialysis buffers failed to improve the recoveries. We concluded that apart from basic instability, S-GT seemed to form aggregates with subcellular structures and other proteins in cell homogenates, leading to inactivation and physical removal by centrifugation and microfiltration.

Anion-exchange chromatography of ammonium sulfate-precipitated or dialyzed crude extracts proved to be effective and consistent, increasing the specific activity 4- to 5-fold, with 80 to 90% recovery. Active pooled fractions were subjected to preparative IEF, but all the S-GT was precipitated and inactivated near its pI. Preparative native PAGE with Coomassie staining revealed very few distinct protein bands, with the bulk of the proteins being distributed evenly throughout the gel (Fig. 1A). In the area of S-GT activity,

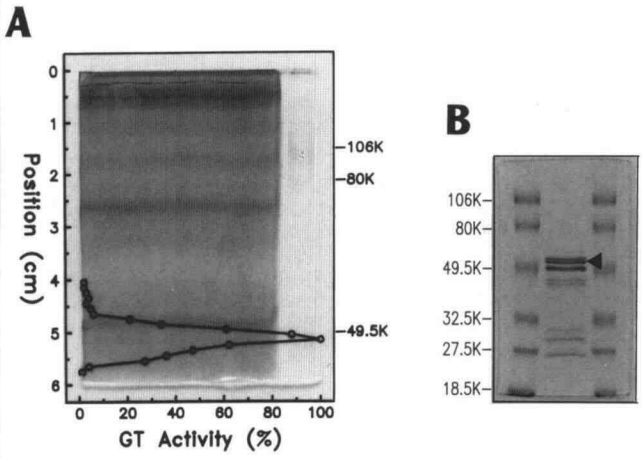


**Figure 1.** Preparative native PAGE (A) and analytical SDS-PAGE of S-GT (B). A, Samples for preparative native PAGE (approximately 3 mg protein/gel) were preprocessed by precipitation of extracts with 40 to 70% saturated ammonium sulfate and FPLC anion-exchange chromatography (Mono Q). Progress of runs was monitored with visible prestained protein markers (numbers to the right). One-half of a gel was Coomassie stained. A 3-mm-wide vertical strip was cut from the unstained section for locating S-GT activity. The activity band was excised from the remainder of the gel and the proteins were electroeluted. B, SDS-PAGE (10%), Coomassie stained. Outer lanes, Prestained markers; center lane, proteins electroeluted from the S-GT activity region of a native PAGE gel (see A). Arrow indicates position of S-GT, as established by immunoprecipitation (see Fig. 2).

low levels of protein without any resolution were generally found. However, proteins electroeluted from the peak activity area (10–20% activity recovery, approximately 200-fold purification) resolved very well in SDS-PAGE (Fig. 1B). Unfortunately, too many contaminating proteins, including a major one migrating at 49.5K, precluded identification of the S-GT protein band. The protein band (tight doublet) migrating at approximately 55K presumably contained S-GT, according to later identification with the aid of antibodies. Its doublet nature probably reflected the presence of a limited-degradation product of S-GT generated during purification.

We concluded that purification of S-GT using conventional chromatographic and electrophoretic means was complicated further by copurification with an abundance of proteins of very similar physico-chemical properties. Persistent copurification of S-GT with desulfoglucosinolate sulfotransferase, on a wide variety of chromatographic media, was recently reported (Jain et al., 1990b).

To purify S-GT to homogeneity repeatedly and in amounts sufficient for protein characterization, we explored immunoaffinity techniques involving monoclonal antibodies. Screening of hybridoma cells for monoclonal antibody production had the advantage that partially purified S-GT could be used as an antigen.

### Immunoaffinity Purification

The monoclonal antibody developed against S-GT did not inactivate or precipitate the enzyme in free solution. If agarose-goat anti-mouse immunoglobulin antibody was added to a mixture of monoclonal antibody and S-GT, a maximum of about 50% enzyme activity was precipitated. Complete removal was realized if the monoclonal antibody was first adsorbed onto the agarose-goat antibody complex and all free antibody was removed by washing.

Quantitative immunoprecipitation of S-GT activity facilitated the identification of a corresponding protein band in SDS-PAGE (Fig. 2). Concomitant removal of S-GT and a protein band at 55K in SDS-PAGE was demonstrated with an electrophoretically enriched enzyme sample. In contrast, protein disappearance could not be visualized with a crude extract and anion-exchange fractionated enzyme, since too much contaminant protein was present that comigrated with S-GT in SDS-PAGE.

The monoclonal antibody did not recognize S-GT protein in SDS-PAGE immunoblots, because its epitope topography likely involved secondary structure that was present only in native enzyme. A good signal in native PAGE immunoblots could be generated with the monoclonal antibody, but S-GT had to be loaded in quantities large enough (>7.5 μg/lane) for sufficient protein to survive the denaturing conditions of electroblotting. In practice, only immunopurified S-GT preparations contained enough protein to yield signals in native PAGE immunoblots.

Preparative immunoaffinity precipitation of S-GT from crude cauliflower extracts was very effective (Fig. 3). It was quantitative if excess agarose antibody complex was used. Process variables used in purification included variations in

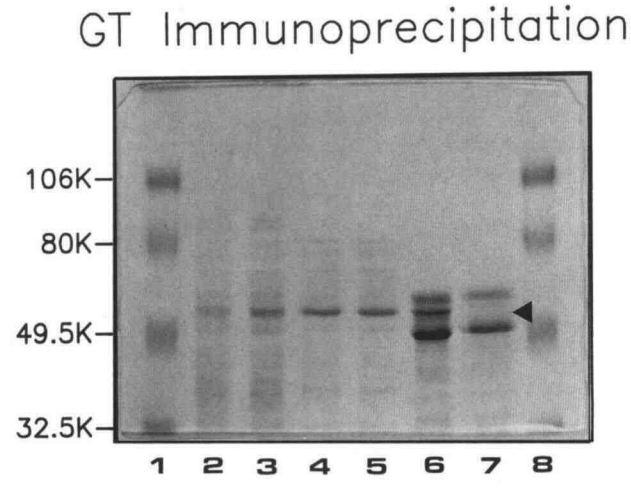


**Figure 2.** Position identification of S-GT protein in SDS-PAGE by immunoprecipitation (arrowhead). Monoclonal antibody was adsorbed to agarose-goat anti-mouse IgG antibody (20 μL) and added to enzyme samples (50 μL), mixed gently for 4 h at 4°C, and centrifuged (1 min at 12,000g). The supernatants were assayed from S-GT activity to ensure 95 to 100% activity removal and subjected to SDS-PAGE. Lanes 1 and 8, Prestained markers; lane 2, crude cauliflower extract; lane 3, same as lane 2 after immunoprecipitation; lane 4, pooled S-GT fractions of anion-exchange-chromatographed crude extract; lane 5, same as lane 4 after immunoprecipitation; lane 6, S-GT electroeluted from preparative native PAGE of anion-exchange-chromatographed crude extract; lane 7, same as lane 6 after immunoprecipitation.

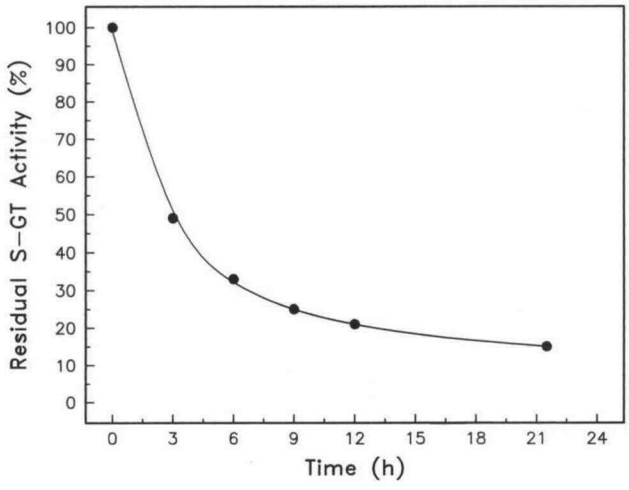


**Figure 3.** Time course of preparative immunoaffinity precipitation of S-GT activity. Agarose-monoclonal antibody complex (1.8 mL) was incubated with 350 mL of crude extract (437 mg of protein from about 500 g of cauliflower tissue). At the indicated times activity was determined in the supernatant of 1-mL aliquots.

enzyme concentrations and a gradual degeneration of the agarose antibody complex over a maximum useful lifetime of eight cycles of enzyme adsorption and elution. A batchwise process for immunopurification was preferred over the more widely used technique of immunoaffinity chromatography (Weselake and Jain, 1992), since the high ratio of enzyme volume to affinity matrix volume would have required much longer adsorption times. The process was also simple to perform, and exposure to the denaturing eluent (triethylamine, pH 11.5) could be limited to a few minutes.

**Table II.** *Purification of thiohydroximate S-glucosyltransferase from cauliflower florets*

| Purification Step | Total Activity[a] | Total Protein[a] | Specific Activity | Yield[b] | Purification Factor[b] |
|---|---|---|---|---|---|
| | *units* | *mg* | *units mg$^{-1}$ protein* | % | |
| Crude extract | 21,000 | 913 | 23 | 100 | 1 |
| Dialysis/microfiltration | 13,000 | 437 | 30 | 60 | 1.3 |
| Immunopurification | 1,950 | 0.22 | 8,800 | 9 | 380 |
| Native PAGE/electroelution | 290 | 0.09 | 3,200 | 1.4 | 140 |

[a] Typical values for 0.5 kg of tissue processed. [b] Values are based on S-GT activity in crude extract.

Native PAGE separation of proteins eluted from the agarose-antibody enzyme complex showed two main bands, one coinciding with S-GT activity (Fig. 4). The S-GT band was relatively broad, likely due to the presence of isoenzymes of different pI values as well as partial denaturation caused by the harsh conditions needed for antigen desorption. The second protein migrating at about 85K apparently was removed from crude extracts through binding to S-GT and not to the antibody directly. It would be of interest to know whether this protein-protein complex plays a physiological role or is an artifact of cell homogenization.

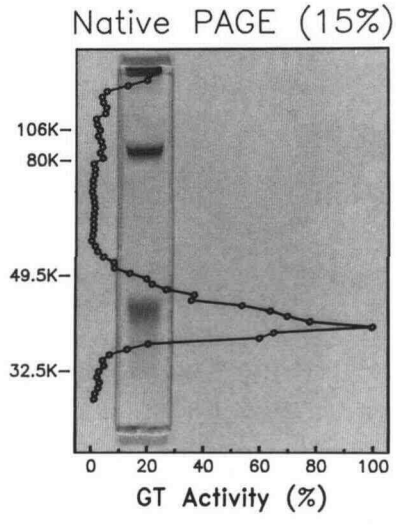


**Figure 4.** Separation and verification of immunopurified S-GT by native PAGE. Preparative native PAGE gels were loaded with immunopurified protein (approximately 100 μg/gel). Progress of runs was monitored with visual prestained markers (numbers to the left). Vertical strips of 3 mm were cut from the sides and center of the gel for Coomassie staining and locating S-GT activity. The activity region was excised from the remainder of the unstained gels and S-GT was recovered by electroelution.

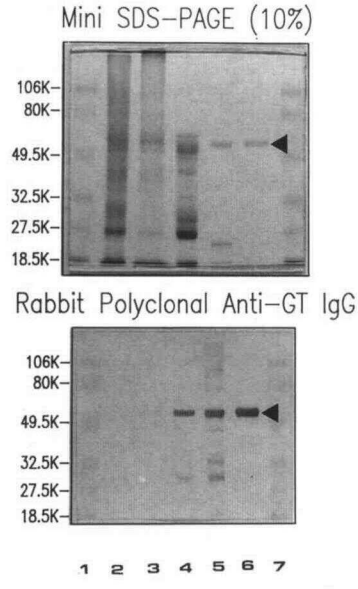


**Figure 5.** SDS-PAGE and immunoblot analysis of S-GT. Lanes 1 and 7, Prestained markers; lane 2, crude cauliflower extract; lane 3, pooled fractions of anion-exchange-chromatographed crude extract; lane 4, electroeluted S-GT from preparative native PAGE of anion-exchange-chromatographed crude extract; lane 5, immunopurified S-GT; lane 6, electroeluted S-GT from preparative native PAGE of immunopurified S-GT. The immunoblot was probed with polyclonal antibodies.

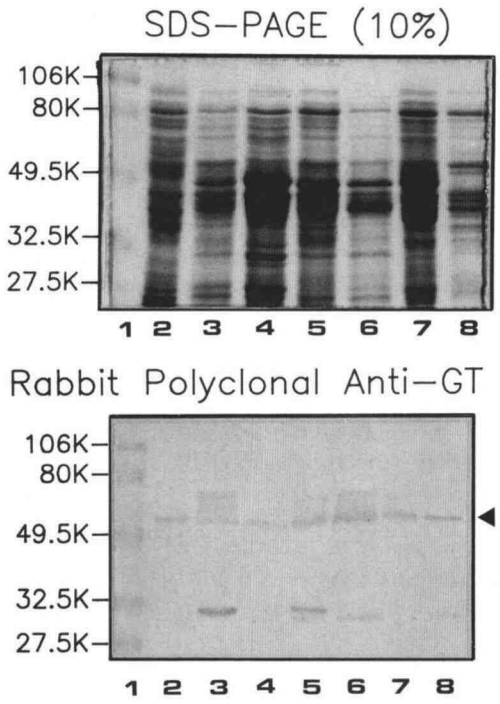


**Figure 6.** Immunoblot analysis of proteins from crucifers with polyclonal S-GT antibodies. SDS-PAGE (top) and immunoblot (bottom). All samples were enriched for S-GT by precipitation with 30 to 70% saturated ammonium sulfate and Superose 12 gel filtration. Lane 1, Prestained markers; lane 2, *B. oleracea* (broccoli) florets; lane 3, *B. campestris* (cv R-500) seedlings; lane 4, *B. napus* (cv Westar) seedlings; lane 5, *B. juncea* (cv Cutlass) seedlings; lane 6, *B. carinata* (PBI 5) seedlings; lane 7, *B. nigra* (rapid cycling) seedlings; lane 8, *S. alba* (cv Gisilba) seedlings.

## S-GT Protein Properties

Homogeneous S-GT was routinely prepared by either preparative native PAGE, SDS-PAGE, or FPLC anion-exchange chromatography of immunopurified proteins. The overall purification protocol with native PAGE as the final step is summarized in Table II. The most striking aspect of the procedure is that the immunopurification step yielded relatively little protein (0.05% recovery) but at a purity as high as 50%. The harsh elution conditions caused denaturation/inactivation of a major fraction of the S-GT enzyme population. Similarly, inactivation was extensive during the final native PAGE/electroelution step, resulting in a very low activity yield and an apparent declining purification factor (Table II). Nevertheless, the end result was protein homogeneity, even though the specific activity was poor.

The S-GT protein had an $M_r$ of 55,500 as determined by SDS-PAGE and 57,600 by FPLC gel filtration (Superose 12) of homogeneous active enzyme (data not shown). Thus, the enzyme appears to be a monomer comprising a single polypeptide. Analytical IEF revealed two protein species of pI 4.80 and 4.95 (data not shown). These $M_r$ values and pI values were both significantly higher than the values recently reported for the enzyme from *B. napus* seedlings (Reed et al., 1993). The reason for the discrepancy is not clear but may be related to differences in the plant species used as enzyme sources. Second, the long purification procedure for the *B. napus* enzyme may have generated a lower mol wt artifact due to limited degradation. Furthermore, its complete homogeneity and identity could not be verified unequivocally due to the small quantities available.

The S-GT enzyme from cauliflower did not appear to be glycosylated as determined with the digoxigenin glycan immunodetection kit (Boehringer) using ferritin as a positive control (results not shown).

## Polyclonal Antibody Specificity

Rabbit serum raised against denatured homogeneous S-GT was used in SDS-PAGE immunoblot analysis of cauliflower enzyme preparations (Fig. 5). The antibody appeared to be very specific for S-GT; no cross-reactivity with other proteins was detected in crude extracts or after anion-exchange chromatography (4- to 5-fold purification). The latter gave a weak S-GT signal at approximately 55K, whereas no signal was detected with crude extracts, confirming the very low concentration in the source tissue. Strong S-GT signals in electrophoretically and immunoaffinity purified S-GT were accompanied by some cross-reactivity at positions of faster migration, probably due to degradation products. In particular, the signal at approximately 29K seemed to represent a degradation product, since it reappeared in previously homogeneous samples of S-GT during storage.

Polyclonal antibodies coupled to agarose did not precipitate S-GT activity, suggesting that in the native enzyme all epitopes (contiguous amino acids) were concealed by secondary

**Table III.** *Binding specificity of monoclonal antibody to thiohydroximate S-glucosyltransferases of Cruciferae species*

Triangle depicts relationship between diploid and amphidiploid species; *n* is chromosome number

| | |
|---|---|
| *Sinapis alba* (cv Gisilba) | 0%[a] |
| *Sinapis arvensis* (AC 8157, wild mustard) | 0% |
| *Descurania sophia* (flixweed) | 0% |
| *Capsella bursa-pastoris* (shepherd's purse) | 0% |
| *Arabidopsis thaliana* (cv Columbia) | 0% |
| *B. nigra* (*n* = 8) (rapid cycling) | 0% |
| *B. carinata* (*n* = 17) (PBI 5) | ~50% |
| *B. juncea* (*n* = 18) (cv Cutlass, allyl type)[b] (AC 60143, butenyl type)[b] | ~50% |
| *B. oleracea* (*n* = 9) (cauliflower/broccoli) | |
| *B. napus* (*n* = 19) (cv Westar) | 100% |
| *B. campestris* (*n* = 10) (cv R-500) | 100% |
| *B. alboglabra* (AC 5402) | |
| *B. incana* (AC 8207) | |
| *B. villosa* (AC 3026) | |
| *B. cretica* (AC 8006A) | |
| *B. hilarionis* (AC 8010) | |
| *B. macrocarpa* (AC 3022) | |
| *B. montana* (AC 8096) | 100% |

[a] Percent of total S-GT activity removed from plant extracts by agarose-monoclonal antibody complex. [b] Allyl or 3-butenyl glucosinolate is predominant.

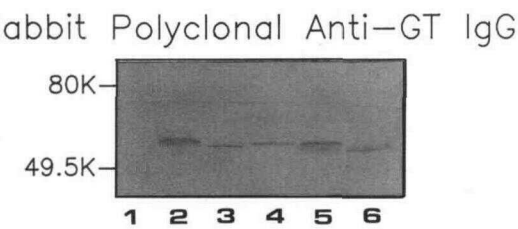


**Figure 7.** Immunoblot analysis of S-GT isoenzymes from amphidiploid and diploid *Brassica* spp. Unless otherwise indicated, enzyme samples were seedling extracts enriched for S-GT by precipitation with 30 to 70% ammonium sulfate, Superose 12 gel filtration, and Mono Q anion-exchange chromatography. Lane 1, Prestained markers; lane 2, *B. nigra*; lane 3, *B. juncea*, immunoprecipitated isoenzyme; lane 4, *B. juncea*, isoenzyme not precipitated by agarose-monoclonal antibody complex (residual activity approximately 50%); lane 5, *B. juncea*; lane 6, *B. campestris*.

and tertiary structure (Harlow and Lane, 1988). However, native PAGE/polyclonal immunoblot analysis of immunopurified S-GT gave a very strong signal (data not shown), presumably due to unfolded protein molecules that were generated during the preparative and analytical processing.

Determination of species specificity showed that the polyclonal antibodies recognized S-GT from several oilseed, condiment, and vegetable *Brassica* species and *S. alba* (Fig. 6). Whether the cross-reactivity with slower migrating proteins (greater than 55K) is related to S-GT remains to be established. Cross-reactivity with proteins at 29K and 30K in some samples is believed to be due to S-GT degradation products, but this needs to be substantiated. Analysis of the crucifer weed species shepherd's purse and flixweed also showed cross-reacting proteins, with the major one migrating at about 52K (data not shown). Establishing firm information on S-GT recognition outside the *Brassica* and *Sinapis* genera will require enzyme preparations of greater purity.

### Monoclonal Antibody Species Specificity

Potential binding of the monoclonal antibody to the S-GT of several crucifer species was determined with crude tissue extracts in a small-scale precipitation test (Table III). The epitope recognized by the antibody occurred on all S-GT in three *Brassica* species, i.e. *B. oleracea* and its wild interfertile relatives of the same chromosome number, as well as *B. napus* and *B. campestris*. Approximately one-half of the S-GT activity in the amphidiploid species *B. carinata* (Abyssinian mustard) and *B. juncea* (brown mustard) was precipitated, suggesting the presence of isoenzymes with differences in the antibody binding site. This partial precipitation was not caused by substances present in crude extracts; results were verified with S-GT enriched by ammonium sulfate fractionation and anion-exchange or gel-filtration chromatography. The epitope was not present in S-GT of *B. nigra* (black mustard), *S. alba* (white mustard), and some cruciferous weed species. Thus, the genetic origin of the epitope could be traced back to the primary diploid species *B. oleracea* and *B. campestris*. In addition to identical mol wt, this suggests that the S-GT of both species are very similar, encoded by highly homologous genes. However, since S-GT from the amphidiploid *B. napus* was used as antigen in immunization and hybridoma screening (activity precipitation), it cannot be ruled out that similarity is restricted to the epitope.

In SDS-PAGE immunoblot analysis *B. juncea* yielded two S-GT bands, at 55.5K and approximately 57K. Immunoprecipitation removed the 55.5K band, which comigrated with that of *B. campestris* (Fig. 7). The remaining 57K band comigrated with that of *B. nigra*. Thus, the isoenzymes of distinct mol wt of the amphidiploid *B. juncea* could be traced to their genomic origin in the constituent ancestors. It was interesting that their activities were perfectly balanced in the amphidiploids (*B. juncea* and *B. carinata*), suggesting the absence of genome bias in gene expression. Furthermore, the total S-GT activities were similar in diploids and amphidiploids, apparently excluding gene dosage effects.

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