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Communication

Glucosinolate Biosynthesis

SULFATION OF DESULFOBENZYLGLUCOSINOLATE BY CELL-FREE EXTRACTS OF CRESS (*LEPIDIUM SATIVUM* L.) SEEDLINGS

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ABSTRACT

After removal of myrosinase activity by concanavalin A-Sepharose 4B chromatography, cell-free extracts of light-grown cress (*Lepidium sativum* L.) seedlings, catalyzed the sulfation of desulfobenzylglucosinolate (K_m, 0.23 millimolar) to benzylglucosinolate using PAPS (K_m, 1 millimolar) as sulfur donor. Sulfotransferase activity, which was optimal at pH 9.0, was stimulated by $MgCl_2$, $MnCl_2$, β-mercaptoethanol, and dithiothreitol and was inhibited by $ZnSO_4$ and SH-reagents. The enzyme also sulfated desulfoallylglucosinolate to allylglucosinolate (sinigrin) but was inactive towards all phenylpropanoids and flavonoids tested.

The glucosinolates constitute a unique class of secondary plant products which are restricted to certain families of dicotyledonous angiosperms including the Cruciferae, Capparidaceae, Resedaceae, and Moringaceae (11). Originally known as mustard oil glycosides, glucosinolates release volatile isothiocyanates, thiocyanates, and nitriles upon degradation by the endogenous thioglucoside glucohydrolase myrosinase. The catabolism and toxicology of glucosinolates have been well documented (11, 19), but relatively little is known about the biosynthesis of these sulfur containing metabolites. Isotopic tracer studies have clearly outlined a biosynthetic pathway (Fig. 1) by which amino acids (I) might be converted to the corresponding glucosinolates (VI), with aldoximes (III), thiohydroximates (IV), and desulfoglucosinolates (V) acting as intermediates (2, 15, 18, 21). Confirmation of this pathway at the enzymic level has been limited. Enzyme preparations obtained from several glucosinolate containing species catalyzed the conversion of *N*-hydroxyphenylalanine to phenylacetaldoxime (10). An UDPG-dependent thiohydroximate glucosyltransferase, which catalyzes the glucosylation of phenylacetothiohydroximate to DSBG,[1] has been purified 20-fold from *Tropaeolum majus* leaves (13). It has been postulated that the final step in the pathway involves the sulfation of desulfoglucosinolates (V) by PAPS. This postulate receives strong support from high incorporation rates of desulfoglucosinolates into glucosinolates in many species (4, 17) and from the accumulation of desulfoglucosinolates *in vivo* when potassium selenate (an inhibitor of biological sulfation processes) was administered in plant feeding experiments (12). Although sulfotransferase activity towards desulfoglucosinolates was reported in 1973 by Underhill (unpublished results), further information on the kinetic and molecular properties of sulfotransferases involved in glucosinolate biosynthesis has not been forthcoming and is the subject of this paper.

Light-grown cress (*Lepidium sativum* L.) seedlings, which contain high levels (1.2 mg/g fresh weight) of BGSL and trace amounts of 2-phenethylglucosinolate in the first week following germination (7), provide an excellent system for investigation of glucosinolate biosynthesis. In this paper, we describe a novel sulfotransferase activity in partially purified extracts from cress seedlings which utilizes PAPS in the sulfation of DSBG to BGSL.

MATERIALS AND METHODS

Plant Materials. *Lepidium sativum* L. (No. 5089, Curled Cress) seeds were purchased from Park Seed Co. (Greenwood, SC). Seedlings were raised in an artificial medium containing one part vermiculite and one part Jiffy Mix (W.R. Grace Co., Cambridge, MA) at 21°C under continuous illumination and harvested after 4 d.

Chemicals. 3′-Phosphoadenosine 5′-phospho-[^{35}S]-sulfate was purchased from NEN Products (Boston, MA) and diluted with unlabeled PAPS as required. BGSL was purified from cress seeds by established procedures (14). DSBG and DSA were obtained from their respective glucosinolates by on-column desulfation (14) using *Helix pomatia* sulfatase with subsequent purification by HPLC on an Ultrasphere ODS C18 reverse phase column (10 mm × 25 cm) with 15% acetonitrile as solvent. The sulfatase, unlabeled PAPS, and allylglucosinolate were purchased from Sigma Chemical Co. Phenylacetaldoxime was synthesized following Underhill (16).

Enzyme Purification. All procedures were undertaken at 4°C. Seedlings (10 g) were homogenized using a pestle and mortar with quartz sand and 20 ml 0.1 M Tris-HCl buffer (pH 8.5), containing 20 mM $MgCl_2$, and 10 mM β-mercaptoethanol. The homogenate was filtered through four layers of cheesecloth and centrifuged at 16,000 *g* for 20 min. Endogenous compounds of low mol wt were removed from the supernatant liquid by passage through Sephadex G-25 columns (8.3 × 1.5 cm) which had been previously equilibrated with 50 mM Tris-HCl buffer (pH 8.5), containing 10 mM β-mercaptoethanol, 20 mM $MgCl_2$, and 0.5% (w/v) NaCl. Removal of myrosinase was achieved by passing the filtrate through a Con A-Sepharose 4B column (5 × 1 cm) which was previously equilibrated with the latter buffer. The preparation was finally concentrated 5-fold by ultrafiltration using an Amicon Ultrafiltration Cell model 52 (PM 10 membrane) and an Amicon B15 Solute Concentrator and stored with 0.25 M sucrose at −20° C. Under these conditions, the enzyme retained

[1] Abbreviations: DSBG, desulfobenzylglucosinolate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; BGSL, benzylglucosinolate; DSA, desulfoallylglucosinolate; Con A, concanavalin A.

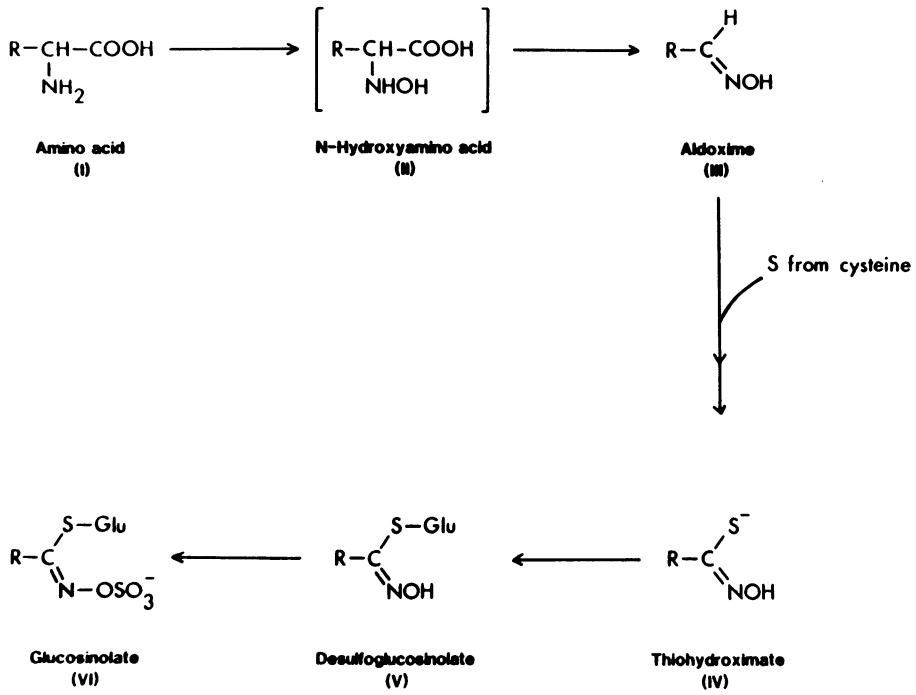

FIG. 1. Proposed pathway for the biosynthesis of glucosinolates.

90% of its original activity after 2 weeks.

Enzyme Assay. The standard assay mixture for sulfotransferase activity contained 10 μmol Tris-HCl buffer (pH 9.0), 34 nmol DSBG, 40 nmol PAPS (containing 100,000 dpm), and 1.1 μmol $MgCl_2$, and up to 0.2 mg protein in a total volume of 110 μl. Control incubations, in which active enzyme was replaced by boiled enzyme or by buffer, were routinely included. After incubation at 30°C for 10 to 60 min, the reaction was terminated by addition of 5 μl of 50% (v/v) acetic acid. Precipitated protein was removed by centrifugation, and an aliquot (90 μl) of the supernatant was applied to Whatman 3MM paper and chromatographed in solvent system I. The product zones were cut out and counted in a Beckman LS 5801 scintillation counter using 10 ml Andersons scintillation cocktail (0.3% [w/v] PPO and 0.02% [w/v] POPOP in xylene-Triton X-114 [3:1, by vol]). All assays were performed in duplicate, and values shown represent the mean of both trials.

Chromatographic Identification of Reaction Product. Identification of the reaction product of DSBG sulfation was made by co-chromatography with an authentic sample of BGSL on Whatman 3MM paper (descending) with solvent systems I and II, and on silica gel GF TLC sheets (Fisher Scientific Co.) with systems III-V. The following solvent systems were utilized: (I) *n*-butanol:pyridine:H_2O, 6:4:3; (II) *n*-butanol:acetic acid:H_2O, 12:3:5; (III) *n*-butanol:*n*-propanol:acetic acid:H_2O, 3:1:1:1; (IV) methylethylketone:ethanol:H_2O, 9:1:2; (V) *n*-propanol:ethyl acetate:H_2O, 7:1:2. BGSL was visualized on paper chromatograms as described in Gmelin and Kjaer (8).

Protein Estimation. Protein estimations were performed by the Bradford procedure (3), using crystalline BSA as standard.

RESULTS AND DISCUSSION

In mammalian tissues, the formation of sulfate esters constitutes a major pathway of metabolism for molecules bearing a hydroxyl or phenolic functional group (9). Following the identification of PAPS as a physiological sulfur donor, sulfotransferase activity has been described towards many acceptor substrates such as bile acids, phenolic steroids, primary and secondary alcohols including hydroxysteroids and phenols (5, 9). Furthermore, the purification and characterization of sulfotransferases showing pronounced substrate specificities have been achieved (6). In plant systems, the synthesis of sulfate esters and sulfolipids is also presumed to involve the intermediacy of PAPS, but little direct evidence is available to support this proposal (1). In 1973, Underhill (unpublished results) showed that extracts from glucosinolate-containing plants utilized PAPS in sulfation of desulfoglucosinolates (18). Recently, Varin *et al.* (20) reported that cell-free extracts of *Flaveria bidentis* and *F. chlorøaefolia* catalyzed the transfer of sulfate groups from PAPS to the hydroxyl groups of several hydroxylated and *O*-methylated flavonols, yielding sulfate esters which are endogenous constituents of *F. bidentis*.

In this publication, we report that protein preparations obtained by homogenizing light-grown cress seedlings in Tris-HCl buffer followed by Sephadex G-25 and Con A-Sepharose chromatography catalyzed the sulfation of DSBG to BGSL at rates of 10 to 20 nmol/h·mg protein. PAPS served as sulfur donor. BGSL production was not observed when DSBG was omitted from the assay mixture nor when active enzyme was replaced by buffer or by enzyme that had been inactivated by boiling for 2 min. Furthermore, removal of endogenous myrosinase activity by passage of the crude preparation through a Con A-Sepharose 4B column was imperative for detection of sulfotransferase activity. The product of the enzymic sulfation of DSBG co-chromatographed with an authentic sample of BGSL using five solvent systems as described in "Materials and Methods." The optimum pH for DSBG sulfation was determined using several different buffers (Fig. 2). Maximum activity was observed around pH 9.0 in Tris-HCl, borate-NaOH, and Na_2CO_3-HCl buffers. Under standard assay conditions at pH 9.0 in Tris-HCl buffer, the rate of DSBG sulfation, catalyzed by 0.2 mg protein, was linear up to 60 min. The extent of sulfation after 60 min at this pH was proportional to the amount of enzyme added up to at least 0.2 mg of protein. The apparent K_m values for DSBG and PAPS were 0.23 and 1 mM, respectively.

In order to determine the effect of $MgCl_2$ and other ions upon sulfotransferase activity, the enzyme was prepared in buffers lacking $MgCl_2$. Enzyme activity was enhanced 9 and 31% by

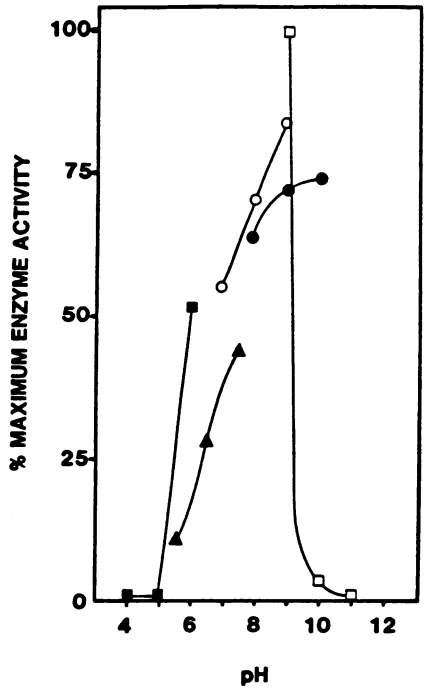

FIG. 2. Effect of pH on PAPS:DSBG sulfotransferase activity. Enzyme activity was assayed as described in "Materials and Methods" but using the following buffers: sodium acetate (■), potassium phosphate (▲), Tris-HCl (○), sodium borate (●), and sodium carbonate-HCl (□).

addition to the assay mixture of 1 mM $MgCl_2$ and $MnCl_2$, respectively. Ca^{2+} ions were without effect, while Cu^{2+} and Zn^{2+} ions at 1 mM concentration inhibited activity by 26 and 63%, respectively. At 10 mM, $MgCl_2$ caused over 80% stimulation of sulfotransferase activity. The enzyme was inhibited 10% by 10 mM EDTA. The flavonol sulfotransferases from *F. bidentis* and *chloraefolia* showed differences in their responses to metal ions (20). While both were stimulated by 1 to 10 mM $MgCl_2$ and potently inhibited by 10 mM $MnCl_2$, the *F. bidentis* enzyme was activated by all other metal ions tested, whereas the *F. chloraefolia* enzyme was inhibited.

The effect of thiol compounds on the activity of sulfotransferase preparations obtained in the absence of β-mercaptoethanol was investigated. Enzyme activity was stimulated 3-fold by 5 mM β-mercaptoethanol; little additional effect was shown by 10 and 15 mM concentrations. DTT (5–15 mM) caused 3- to 4-fold enhancement of activity. In contrast, the enzyme was inhibited 96, 97, and 32%, respectively, by *p*-chloromercuriphenyl sulfonate, *N*-ethylmaleimide, and iodoacetic acid when supplied at 10 mM concentration. The *Flaveria* sulfotransferases exhibited similar behavior in being stimulated by β-mercaptoethanol and DTE and strongly inhibited by SH-group reagents (20).

The *Flaveria* sulfotransferases sulfated hydroxylated and methoxylated flavonols, but were essentially inactive towards flavones and phenylpropanoids (20). The substrate specificity of the *L. sativum* sulfotransferase was investigated by incubating the enzyme at pH 9.0 with several phenylpropanoids, flavonols, and flavonol glycosides at 1 mM concentration. Using an enzyme preparation which sulfated DSBG at a rate of 15 nmol/h·mg protein, no activity was detected by the method of Varin *et al.* (20) towards kaempferol, quercetin, rutin, *p*-coumaric acid, caffeic acid or ferulic acid. Using the standard chromatographic assay method with solvent system I, the enzyme sulfated DSA (1 mM) to allylglucosinolate at a rate of 4.5 nmol/h·mg protein but was inactive towards phenylacetaldoxime (0.1–10 mM), a presumed precursor of BGSL.

In conclusion, this paper, which describes for the first time kinetic properties of a PAPS:DSBG sulfotransferase (EC 2.8.2.-) activity in cell-free extracts from *L. sativum* seedlings, provides strong support for the PAPS-mediated enzymic sulfation of desulfoglucosinolates as the final step in glucosinolate biosynthesis.

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