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Biosynthesis of the Cyanogenic Glucoside Dhurrin in Seedlings of *Sorghum bicolor* (L.) Moench and Partial Purification of the Enzyme System Involved[1]

Barbara Ann Halkier and Birger Lindberg Møller*

Plant Biochemistry Laboratory, Department of Plant Physiology, Royal Veterinary & Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Denmark

ABSTRACT

The cyanogenic glucoside dhurrin is rapidly synthesized in etiolated seedlings of *Sorghum bicolor* (L.) Moench. The dhurrin content of the seedlings increases sigmoidally with the germination time. Shoots of 10 centimeters height contain 850 nanomoles of dhurrin per shoot corresponding to 6% of the dry weight. The biosynthetic activity sharply rises upon germination and reaches a maximum level of 10 nanomoles dhurrin/(hour × shoot) after 48 hours when the shoots are 3 centimeters high. This maximum level is followed by a sharp decline in activity when germination time exceeds 65 hours. Dhurrin and the dhurrin-synthesizing enzyme system are primarily located in the upper part of the etiolated shoot where both are evenly distributed between the coleoptile, the primary leaves and the upper 0.5 centimeter of the first internode including the shoot apex. Dhurrin constitutes 30% of the dry weight of the upper 1.2 centimeter of 10 centimeter high shoots. The seed and root contain neither dhurrin nor the dhurrin-synthesizing enzyme system. The codistribution of dhurrin and the enzyme system throughout the seedling indicates that production and storage sites are located within the same cell. Purification of the dhurrin-synthesizing enzyme by gel filtration or by sucrose gradient centrifugations results in a tenfold increase in specific activity. Further purification is accompanied by a decline in specific activity due to loss of essential components as demonstrated by reconstitution experiments.

High levels of cyanogenic glucosides are found in a number of plants of which several are important food crops (6). Most important in this respect are the tubers of cassava (*Manihot esculenta*) Crantz (4, 7). Inadequate detoxification of cassava products causes cyanide poisoning of millions of people who are dependent on this plant as their major staple food (25). Cyanogenic glucosides accumulate in different plant tissues (*e.g.* seedlings, leaves, tubers, seeds) dependent on the particular plant species (6). A function of cyanogenic glucosides in plants has not been elucidated but they may act as defense compounds. In white clover (*Trifolium repens* L.), the *Ac/ac* locus controls the production of the cyanogenic glucosides linamarin and lotaustralin and plants homozygous for the *ac* allele are acyanogenic (5). Traditional breeding has produced 'low cyanide' cultivars of cassava (11) and sorghum (12), but so far it has not been possible to obtain totally acyanogenic varieties. The polymorphism of cyanogenesis in white clover (5, 14) demonstrates that the cyanogenic character is not of vital importance for normal growth and development of each individual cyanogenic plant. It may therefore be possible to convert a cyanogenic plant like cassava into an acyanogenic form by application of the techniques of molecular biology. A prerequisite for such experiments would be the identification and characterization of the enzymes involved in the biosynthesis of cyanogenic glucosides.

We have chosen sorghum as the model plant for our studies. Seedlings of sorghum (*Sorghum bicolor* [L.] Moench) synthesize the cyanogenic glucoside dhurrin (β-D-glucopyranosyloxy-(*S*)-*p*-hydroxymandelonitrile) derived from L-tyrosine (1). Studies involving labeled precursors and trapping experiments have shown that *N*-hydroxytyrosine, *p*-hydroxyphenylacetaldoxime, *p*-hydroxyphenylacetonitrile, and *p*-hydroxymandelonitrile are intermediates in the pathway (Fig. 1) (20, 22, 24, 27). A microsomal preparation isolated from etiolated seedlings of sorghum catalyzes the conversion of L-tyrosine into *p*-hydroxymandelonitrile, *i.e.* all but the last step in the pathway (20, 22). The dhurrin-synthesizing enzyme system present in the microsomal preparation constitutes a highly organized enzyme system exhibiting catalytic facilitation and thereby providing an efficient mechanism for channeling the flow of carbon from tyrosine into *p*-hydroxymandelonitrile (23). The latter compound is converted to dhurrin by a soluble and specific UDP-glucose glucosyltransferase (24). The present paper reports the changes in the content of dhurrin and the dhurrin-synthesizing enzyme system in etiolated sorghum seedlings during germination. The tissue distribution of dhurrin and its enzyme system as well as procedures for partial purification of the enzyme system are also described.

MATERIALS AND METHODS

Plant Material

Seeds of *Sorghum bicolor* (L.) Moench (hybrid Redland × Greenleaf) were obtained from Seedtec International Inc. (Hereford, TX). After imbibition for 24 h, seeds were germinated between two sheets of gauze stretched over a metal screen placed in a germination tray. This permits easy harvest

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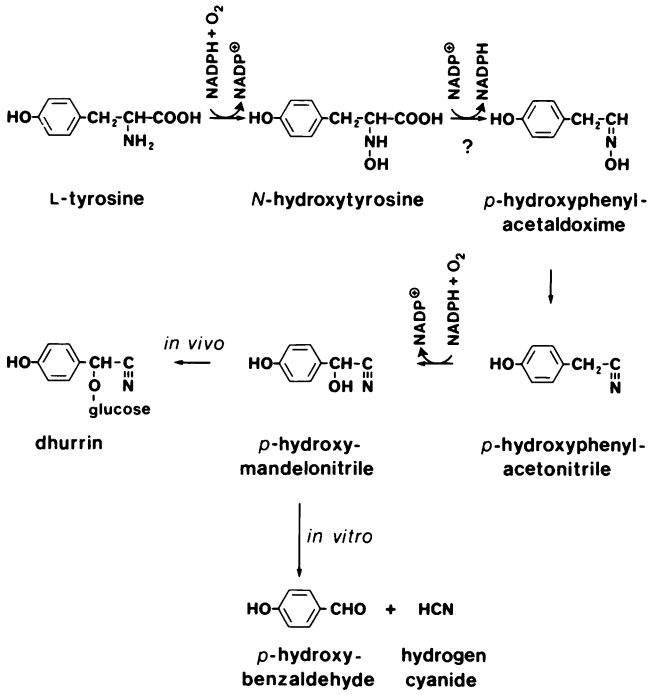

Figure 1. Biosynthetic pathway of the cyanogenic glucoside dhurrin.

of the shoots emerging through the upper gauze layer and prevents contamination with seed coats which are inhibitory to the dhurrin-synthesizing enzyme system. Imbibition and germination were carried out at 28°C and in complete darkness. The transfer of the seeds to the germination trays was carried out under dim green light (Philips TLD36W/17 Green fluorescent tube, light intensity 0.08 μmol/[m^2 × sec]). For determination of dhurrin and the dhurrin-synthesizing enzyme system as a function of germination time, seeds were germinated for 1 to 3 d. Seeds for enzyme purification were germinated 2 d.

Determination of Dhurrin

For determination of the dhurrin content, plant material obtained from a known number of seedlings was weighed and homogenized in liquid nitrogen using a mortar and pestle. The frozen powder was quickly transferred to a precooled cryotube and stored at −80°C. An aliquot of the frozen sample (approximately 5 mg) was withdrawn using a precooled spatula and transferred to a preweighed test tube containing 250 μL 50 mM Mes (pH 6.5) and 0.1 mg emulsin (Almond β-glucosidase type II, Sigma Chemical Co., St. Louis, MO). The test tube was immediately weighed and closed with a silicon septum. Enzymic hydrolysis of dhurrin into *p*-hydroxymandelonitrile was achieved by incubation for 2 h at 30°C. The *p*-hydroxymandelonitrile formed was dissociated into cyanide and *p*-hydroxybenzaldehyde by injection of 40 μL 6 N NaOH and incubation for 1 h at room temperature. A spectrophotometric cyanide assay (see below) permitted quantitative determination of the dhurrin content.

Intact plant segments were used for determination of the tissue distribution of dhurrin. To disrupt the plant tissue, the samples were frozen and thawed three times in the emulsin solution.

Content and Distribution of the Dhurrin-Synthesizing Enzyme System

Freshly harvested shoots or dissected tissue samples (0.1–1.0 g) obtained from a known number of seedlings were weighed and homogenized in a total of 20 mL isolation medium composed of 250 mM sucrose, 100 mM Tricine (pH 7.9), 50 mM NaCl, 2 mM EDTA, 2 mM DTT, and 100 mg polyvinylpolypyrrolidone using a mortar and pestle. The homogenate was centrifuged for 10 min at 10,000*g*. The supernatant was collected with a Pasteur pipette and centrifuged for 1 h at 165,000*g*. The microsomal pellet was resuspended in 1 mL isolation medium and dialyzed overnight against 50 mM Tricine (pH 7.9), 2 mM DTT under a nitrogen atmosphere.

Determination of the Activity of the Dhurrin-Synthesizing Enzyme

The activity of the dhurrin-synthesizing enzyme system was determined as the ability of known aliquots of the dialyzed microsomal preparation to produce cyanide when incubated with saturating amounts of substrate and the cofactor NADPH. Typically, the reaction mixture contained 10 to 100 μL enzyme extract, 125 nmol substrate (tyrosine or *p* hydroxyphenylacetonitrile), 0.3 μmol NADPH, and 75 μmol Tricine in a total volume of 250 μL (pH 7.9). The reaction mixture was incubated for 30 min at 30°C in a septum-covered vial. The enzymic reaction was stopped by injection of 40 μL 6 N NaOH through the septum. Cyanide analysis was carried out after incubation for 1 h at room temperature.

Partial Purification of the Dhurrin-Synthesizing Enzyme System

Microsomal preparations were obtained by a modification of the method of Møller and Conn (22). Etiolated seedlings (approximately 150 g) were harvested and homogenized using a mortar and pestle in 2 volumes (v/w) of 250 mM sucrose, 100 mM Tricine (pH 7.9), 50 mM NaCl, 2 mM EDTA, 2 mM DTT. Polyvinylpolypyrrolidone was added (0.1 g/g fresh weight) prior to homogenization. The homogenate was filtered through 22 μm nylon cloth and centrifuged 20 min at 48,000*g*. The supernatant was centrifuged for 1 h at 165,000*g*. The microsomal pellet was resuspended and homogenized in isolation buffer using a Potter-Elvehjem homogenizer fitted with a Teflon pestle. After recentrifugation and rehomogenization, the homogenate was dialyzed overnight against 50 mM Tricine (pH 7.9), 2 mM DTT under a nitrogen atmosphere.

The microsomal pellet (approximately 50 mg protein) was purified by gel filtration on a column (2.6 × 90 cm) of Sephacryl S-1000 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in 25 mM Tricine (pH 7.9), 2 mM EDTA, 2 mM DTT (flowrate: 25 mL/h). Biosynthetically active fractions were combined (60 mL), and an aliquot (30 mL) was applied to a column (1.6 × 4.5 cm) of 2′,5′-ADP-Sepharose (Pharmacia Fine Chemicals) equilibrated in 100 mM Tricine

(pH 7.9), 2 mM EDTA, 2 mM DTT, 20% glycerol (flowrate: 8 mL/h). The column was washed with equilibration buffer and elution carried out using 10 mM NADPH in the same buffer. After dialysis to remove NADPH, the activity of the dhurrin-synthesizing enzyme system and the protein content of the collected fractions were determined.

In an alternative procedure, the microsomal pellet (approximately 50 mg protein) was applied to six 0.5 to 1.6 M linear sucrose gradients (12 mL) prepared in 20 mM Tricine (pH 7.9), 2 mM EDTA, 2 mM DTT, and centrifuged for 9 h at 157,000*g*. Fractions enriched with respect to the dhurrin-synthesizing enzyme system were combined, diluted with an equal volume of 50 mM Tricine (pH 7.9), 2 mM DTT, and further purified on a 0.5 to 1.0 M linear sucrose gradient using the same experimental conditions as above. For determination of the distribution of the dhurrin-synthesizing enzyme system and of the protein content, each of the sucrose gradients were fractionated into 500 μL aliquots.

Cyanide Determination

Cyanide was determined spectrophotometrically using the chemical reactions described by König (16). The assay involves sequential addition of 50 μL HOAc, 200 μL *N*-chlorosuccinimide (1 g/L)/succinimide (2.5 g/L) and 200 μL pyridine (300 mL/L)/barbituric acid (60 g/L) to the cyanide-containing reaction mixture. The colored complex formed was quantified by dual-wavelength spectrometry with wavelength settings at 585 and 650 nm using an Aminco DW2C spectrophotometer. To compensate for absorbance resulting from endogenous cyanide or nonenzymically formed cyanide, reaction mixtures containing no NADPH were also prepared and measured.

The *N*-chlorosuccinimide/succinimide reagent was prepared by initial solubilization of succinimide and subsequent addition of solid *N*-chlorosuccinimide as recommended by Lambert *et al.* (18). The *N*-chlorosuccinimide/succinimide and the pyridine/barbiturate reagents were prepared directly in air-tight Oxford dispensors and were stored at 4°C. These precautions prolonged the stability of the reagents from a few days to more than a month.

Additional Analytical Procedures

The dry weight of tissue samples was determined after freeze-drying followed by incubation for 2 d in an exsiccator. Analytical SDS-PAGE was performed using 8 to 25% linear gradient gels prepared as described by Fling and Gregerson (8). After electrophoretic fractionation, the gels were stained with Coomasie brilliant blue R250 or alkaline silver (21). Protein concentrations were determined using the method of Bradford (3).

RESULTS AND DISCUSSION

Quantification of Dhurrin and the Dhurrin-Synthesizing Enzyme System

Sorghum seedlings contain a specific β-glucosidase capable of converting dhurrin into *p*-hydroxybenzaldehyde, cyanide, and glucose. The β-glucosidase is brought into contact with the dhurrin upon maceration of the plant tissue (6). The cyanide formed is volatile and therefore partially lost during the homogenization procedure used as the initial step in the preparation of microsomes (see "Materials and Methods"). Residual amounts of dhurrin and cyanide are removed by dialysis. The activity of the dhurrin-synthesizing enzyme system can then be assayed *in vitro* as the conversion rate of the substrates tyrosine or *p*-hydroxyphenylacetonitrile into *p*-hydroxymandelonitrile (Fig. 1). *p*-Hydroxymandelonitrile dissociates quantitatively into *p*-hydroxybenzaldehyde and cyanide, the end products in the microsomal reaction mixtures. Cyanide is determined by a spectrophotometric assay and the reaction is carried out in septum-covered vials to prevent any loss of cyanide.

To determine the amount of endogenous dhurrin present in the plant material, the action of the endogenous β-glucosidase and concomitant loss of dhurrin (as cyanide) during homogenization is prevented by homogenization and subsequent storage of the plant material in liquid nitrogen. Transfer of aliquots of frozen plant material into septum-covered vials containing exogenously added almond β-glucosidase ensures rapid and quantitative conversion of dhurrin into *p*-hydroxybenzaldehyde, cyanide, and glucose. Consequently, the content of dhurrin can be quantified as cyanide.

The method of Lambert *et al.* (18) has previously been used to determine the cyanide content of biological reaction mixtures. In those experiments, an overnight incubation of septum-covered vials was required to permit diffusion of hydrogen cyanide from an acidified reaction mixture to a suspended center-well containing 1 N NaOH (22, 23). In this study, a modified procedure was developed, in which the amount of cyanide is measured directly in the reaction mixture. After enzymic incubation, a NaOH-solution is injected into the reaction mixture to: (a) stop the enzymic reaction, (b) ensure complete dissociation of *p*-hydroxymandelonitrile into *p*-hydroxybenzaldehyde and cyanide, (c) trap hydrogen cyanide from the gas phase of the vial into the aqueous phase. The cyanide content of the aqueous phase is then quantified by means of a scaled-down dual-wavelength spectrophotometric assay as described in "Materials and Methods." The assay permits accurate determination of as little as 1 nmol of cyanide.

Tissue Distribution of Dhurrin and the Dhurrin-Synthesizing Enzyme System

The dhurrin is located in the upper part of the shoot which contains the coleoptile, the primary leaves, and the upper 0.5 cm of the first internode including the shoot apex (apical meristem). A typical set of data for the distribution of dhurrin in a single 8 cm high etiolated sorghum seedling is shown in Figure 2. The sorghum content in the seed and in the root is negligible. The dhurrin-synthesizing enzyme system is also primarily located in the upper part of the etiolated sorghum seedling and is absent from the seed and the root (Fig. 2, inset). Being the major site of synthesis and storage, the upper part of the seedling was further dissected into the coleoptile, the primary leaves and the upper 0.5 cm of the first internode including the shoot apex. Each of these tissues was found to contain about equal amounts of the enzyme system as well as of dhurrin (data not shown). The codistribution of dhurrin

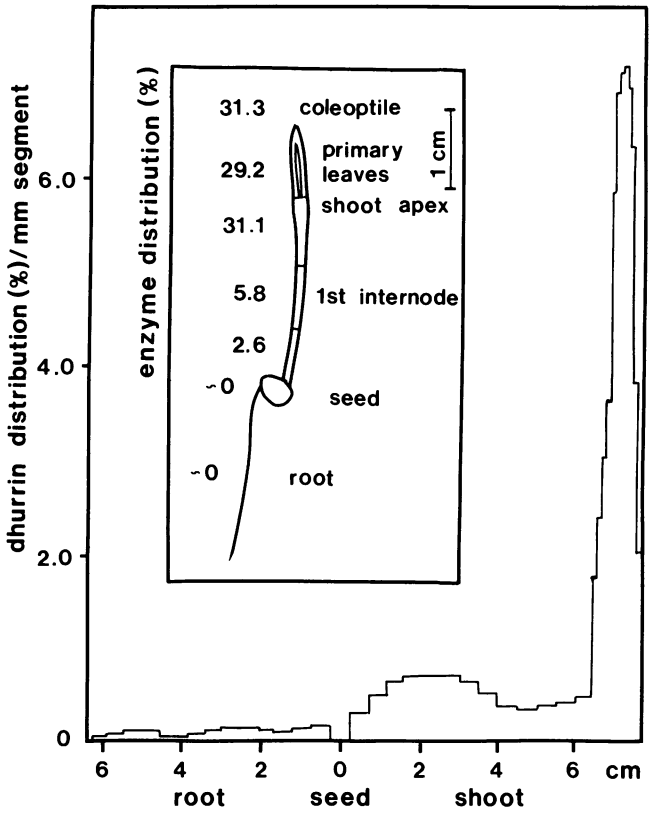

Figure 2. Distribution of dhurrin and the dhurrin-synthesizing enzyme system within an etiolated sorghum seedling. An 8 cm high seedling was dissected into 1 and 5 mm segments as indicated by the bar width and the dhurrin content of each segment was quantified. Inset, Eighty 3-cm-high seedlings were dissected as indicated on the figure. Microsomes were prepared from each of the five segments of the shoot and from seeds and roots and analyzed for their content of the dhurrin-synthesizing enzyme system.

and the enzyme system throughout the seedling suggests that storage and production sites are located within the same cell.

The distribution and catabolism of dhurrin in the two leaf-blades of 6-d-old, light-grown sorghum seedlings have been studied by Kojima *et al.* (17). Essentially all dhurrin was located in the epidermal cell layer whereas the β-glucosidase involved in dhurrin catabolism was localized primarily in the mesophyll tissue and bundle sheath strands. Kojima *et al.* (17) could not determine the localization of the dhurrin-synthesizing enzyme system since the microsomes prepared from the light-grown seedlings were not biosynthetically active. Compartmentalization of dhurrin and β-glucosidase into different tissues provides one mechanism whereby dhurrin can be protected from enzymic degradation. However, in the present study with etiolated seedlings of sorghum, the compartmentalization appears to proceed at the intracellular level in agreement with evidence suggesting that dhurrin is stored in the vacuole (26), whereas β-glucosidases are generally not present in the vacuole (9).

Dhurrin and the Dhurrin-Synthesizing Enzyme System during Germination

The fresh and dry weight of shoots of etiolated sorghum seedlings as a function of germination time are shown in Figure 3. The height of the shoots as a function of germination time is also indicated. Data from one typical experiment on the changes in the content of dhurrin and the dhurrin-synthesizing enzyme system per seedling as a function of germination time are shown on Figure 4. The dhurrin content in the shoot shows a sigmoidal increase with germination time. The increase is linear when depicted as a function of the shoot height (plot not shown). The activity of the dhurrin-synthesizing enzyme system in the shoot reaches a maximum value of 10 nmol HCN/(h × shoot) after 48 h when the shoots are 3 cm high (Fig. 4). This maximum level is followed by a sharp decline in activity when the germination time exceeds 65 h corresponding to a shoot height of 7 cm. From the experimentally determined activity of the dhurrin-synthesizing enzyme system, a corresponding value for the dhurrin content of the shoot at a given time can be calculated. The calculated dhurrin content shows a sigmoidal increase with germination time as is also observed with the experimentally determined dhurrin content (Fig. 4). The calculated values equal 45% of the experimentally determined values. The isolation of the microsomal system containing the dhurrin-synthesizing enzyme system involves initial homogenization and subsequent fractionation and is unlikely to result in a quantitative recovery of the enzyme system. The experimentally determined content of the dhurrin-synthesizing enzyme system may therefore be underestimated. Thus, the difference between the experimentally determined and the calculated dhurrin content could reflect the incomplete recovery of the enzyme system during isolation. Alternatively, the difference could indicate that the dhurrin-synthesizing enzyme system *in vivo* operates at a tyrosine concentration close to the K_m value (0.03 mM) (22) and not at a saturating substrate concentration as used

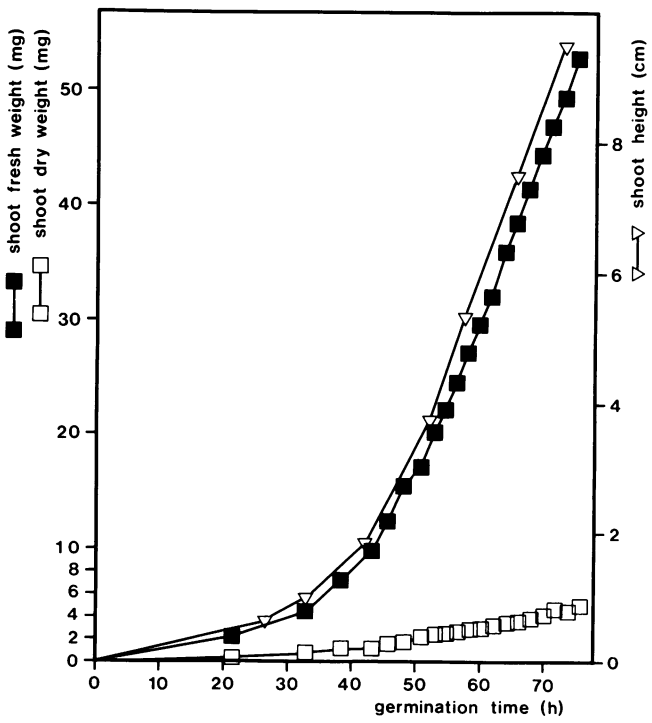

Figure 3. Fresh weight, dry weight, and height of etiolated sorghum shoots as a function of germination time.

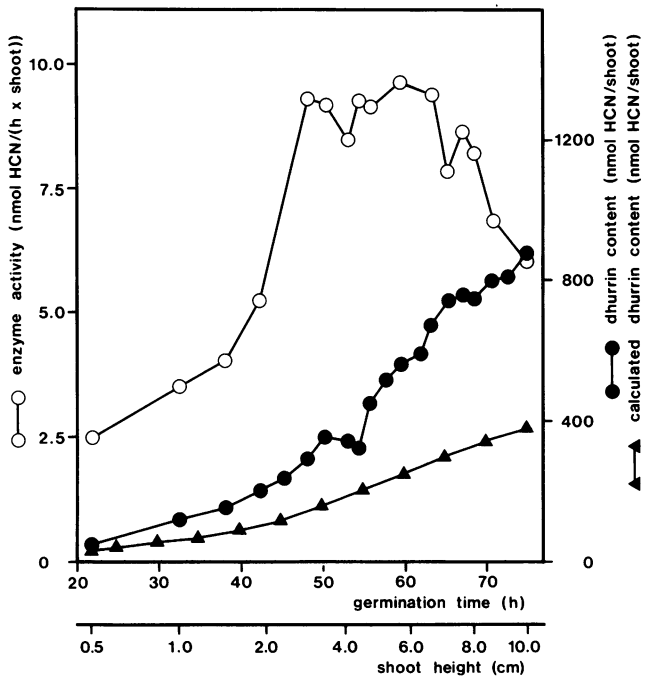

Figure 4. Content of dhurrin and of the dhurrin-synthesizing enzyme system per sorghum shoot as a function of germination time. Duplicate samples (each containing approximately 25 shoots) were collected and used for the determination of dhurrin and the dhurrin-synthesizing activity, respectively, as described under "Materials and Methods." Calculated values for the dhurrin content based on the experimentally determined activity of the dhurrin-synthesizing enzyme system are also presented. The second abscissa indicates the relationship between the height of the shoot and the germination time as derived from Figure 3.

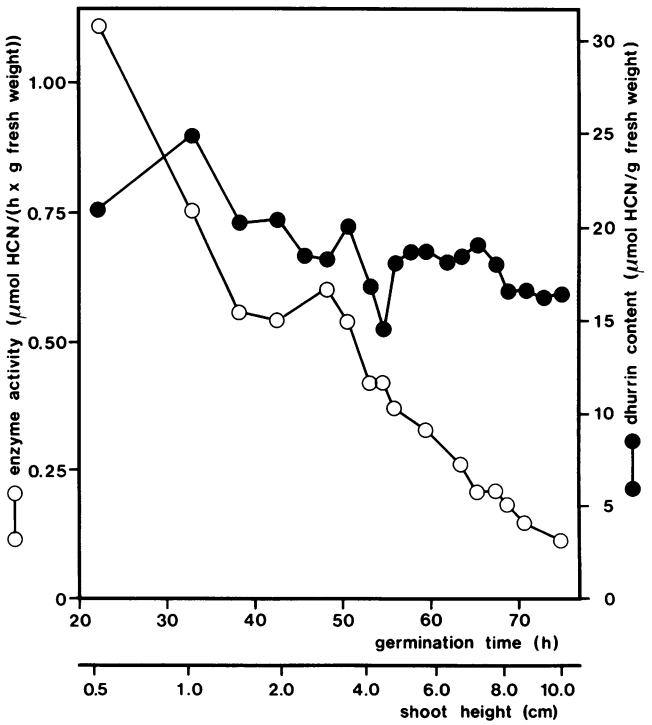

Figure 5. Content of dhurrin and of the dhurrin-synthesizing enzyme system per g fresh weight of etiolated sorghum shoots. The second abscissa indicates the relationship between the height of the shoot and the germination time as derived from Figure 3.

in the *in vitro* assays. In conclusion, the calculation shows that the dhurrin-synthesizing enzyme system *in vivo* operates at or above its K_m value and that the turnover rate of dhurrin is negligible during the germination period examined. It is remarkable that the efficient synthesis of dhurrin proceeds during a time period in which there is a simultaneous high demand for tyrosine and its precursors for synthesis of proteins, lignins, esters of C6-C3 phenolic acids, and flavonoids (28).

The dhurrin content per gram shoot (fresh weight) shows a weak decline with increasing germination time, but is typically around 18 μmol dhurrin equivalent to 0.5% of the fresh weight and 6% of the dry weight (Fig. 5). Akazawa *et al.* (1) have previously investigated the content of dhurrin in etiolated sorghum seedlings and report a content of 18.5 μmol dhurrin/g seedling (fresh weight) in shoots of 9 to 10 cm. As shown by our data (Fig. 2), 59% of the dhurrin content of the seedling is located in the upper 1.2 cm of the shoot. The dhurrin concentration in this region of 10 cm shoots consequently reaches levels as high as 2.5% of the fresh weight or 30% of the dry weight.

Partial Purification of the Dhurrin-Synthesizing Enzyme System

The last step in the biosynthesis of dhurrin (Fig. 1) is catalyzed by a soluble UDP-glucose glucosyl-transferase which has been purified from sorghum (24) and flax (*Linum usitatissimum* L.) (10). The prior steps are catalyzed by a membrane-associated multienzyme system (23). Neither the intact enzyme system nor any of the enzyme components of this complex have been isolated. Some of the polypeptides associated with the dhurrin-synthesizing enzyme system may be extrinsic and therefore easily lost during membrane fractionation. The fractions obtained were therefore routinely tested for biosynthetic activity using two substrates: tyrosine, the first substrate of the pathway, and *p*-hydroxyphenylacetonitrile, the immediate precursor of *p*-hydroxymandelonitrile. Using both substrates, enzyme activity was measured as the production of HCN.

The dhurrin-synthesizing enzyme system is associated with the microsomal pellet obtained by differential centrifugation. The initial centrifugation at 48,000*g* efficiently removes membranes derived from mitochondria and proplastids. The dhurrin-synthesizing enzyme system was further purified by gel filtration of the microsomal preparation on Sephacryl S-1000. The fractions containing the dhurrin-synthesizing enzyme system were turbid and yellow and were clearly separated from the major part of the proteins as evidenced from the A_{254} profile (Fig. 6). The specific activity of the active fractions using tyrosine as substrate was 1140 nmol HCN/(h × mg protein) compared to a value of 290 nmol HCN/(h × mg protein) for the microsomal preparation. The V_{max} values obtained with the crude microsomal preparations earlier used was 110 nmol HCN/(h × mg protein) (22) due to a less discriminative initial centrifugation step used for their isolation.

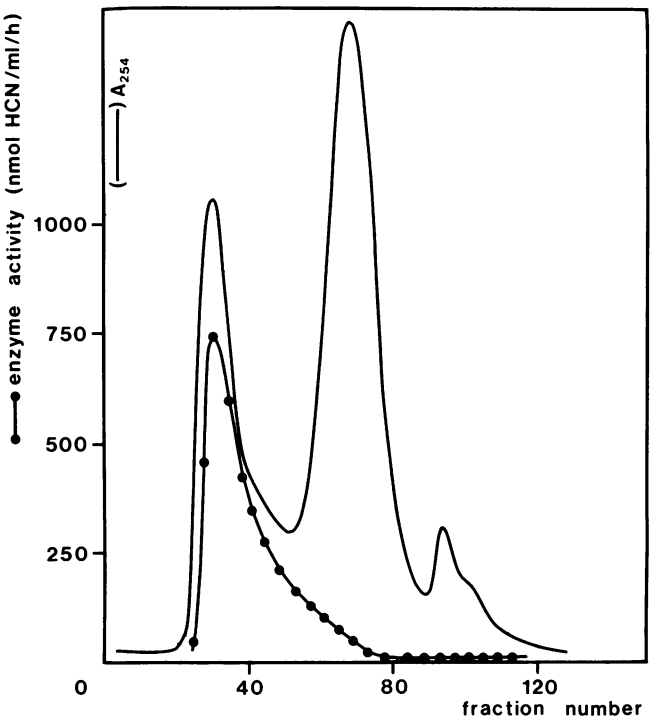

Figure 6. Purification of the dhurrin-synthesizing enzyme system by gel filtration on Sephacryl S-1000. The elution of proteins was monitored at 254 nm. The activity of the dhurrin-synthesizing enzyme system was measured in every fourth fraction using tyrosine as substrate.

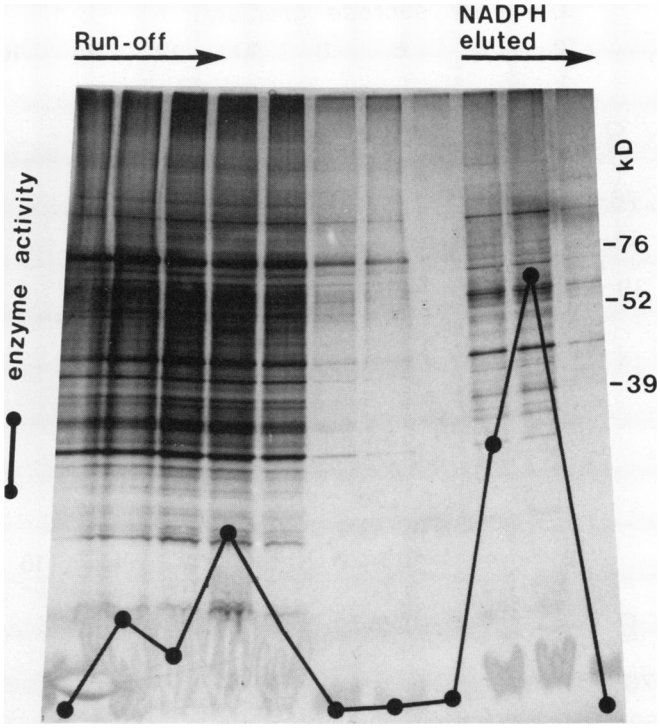

Figure 7. Purification of the dhurrin-synthesizing enzyme system by affinity chromatography on 2′,5′-ADP Sepharose. Biosynthetically active fractions from the Sephacryl S-1000 column were applied to a 2′,5′-ADP Sepharose column. Elution was carried out with an NADPH-containing buffer as described in "Materials and Methods." The polypeptide composition of each fraction was analyzed by SDS-PAGE. The activity profile as obtained by analysis of aliquots of each fraction using *p*-hydroxyphenylacetonitrile as substrate, is superimposed on the silver stained electrophoretogram.

Several steps in the conversion of tyrosine to *p*-hydroxymandelonitrile are hydroxylation reactions dependent on NADPH and molecular oxygen (Fig. 1). The biosynthetically active fractions from the Sephacryl S-1000 column were therefore further purified by affinity chromatography using 2′,5′-ADP-Sepharose. Polypeptides responsible for the biosynthetic activity were bound to the column and could be eluted with an NADPH-containing buffer (Fig. 7). The polypeptide composition of the NADPH-eluted polypeptides was clearly different from that of the nonbound material as monitored by silver stained SDS-polyacrylamide gels (Fig. 7). The specific activity of the NADPH-eluted material using *p*-hydroxyphenylacetonitrile as substrate was 370 nmol HCN/(h × mg protein) whereas that of the nonbound material was 100 nmol HCN/(h × mg protein). However, the specific activity of the sample applied to the 2′,5′-ADP Sepharose column was 1100 nmol HCN/(h × mg protein). Similar results were obtained using tyrosine as substrate. Both tyrosine and *p*-hydroxyphenylacetonitrile serve as substrates for NADPH-dependent mono-oxygenases, which themselves may consist of several polypeptides (29). The resulting decrease in specific activity possibly reflects dissociation of essential components from the enzyme system.

The dhurrin-biosynthesizing enzyme system was also purified by sucrose gradient centrifugations (Fig. 8). Fractionation of the microsomal preparation on a 0.5 to 1.6 M linear sucrose gradient produced a whitish (d = 1.088 g cm^{-3}) and a yellowish (d = 1.094 g cm^{-3}) turbid band at the top of the gradient above a diffuse clear yellow zone (average d = 1.111 g cm^{-3}) containing the highest concentration of protein. Using tyrosine as a substrate, the proteins in the whitish band near the top of the gradient showed the highest specific activity and were further purified on a 0.5 to 1.0 M sucrose gradient. On this gradient, the proteins responsible for the biosynthetic activity were positioned below the major protein band (Fig. 8). The specific activity of the active fractions from these gradients varied between 800 and 2800 nmol HCN/(h × mg protein). The biosynthetically active fractions are clearly enriched in polypeptides with molecular masses in the region 50 to 80 kD (Fig. 8), but the dhurrin-synthesizing activity may be associated with some of the less dominant polypeptides. The polypeptide composition resembles that obtained by S1000-Sephacryl gel filtration. In contrast to the S1000-Sephacryl preparation, the preparation obtained by sucrose gradient centrifugations is colorless and clear and therefore useful for spectroscopical characterization.

Attempts have also been made to purify the dhurrin-synthesizing enzyme system by ion exchange chromatography and hydrophobic interaction chromatography. A general feature of these studies has been that purification of the enzyme system beyond the levels described above was not accompanied by a corresponding increase in specific activity. This is at least partially due to dissociation of essential components from the enzyme system as evidenced by reconstitution ex-

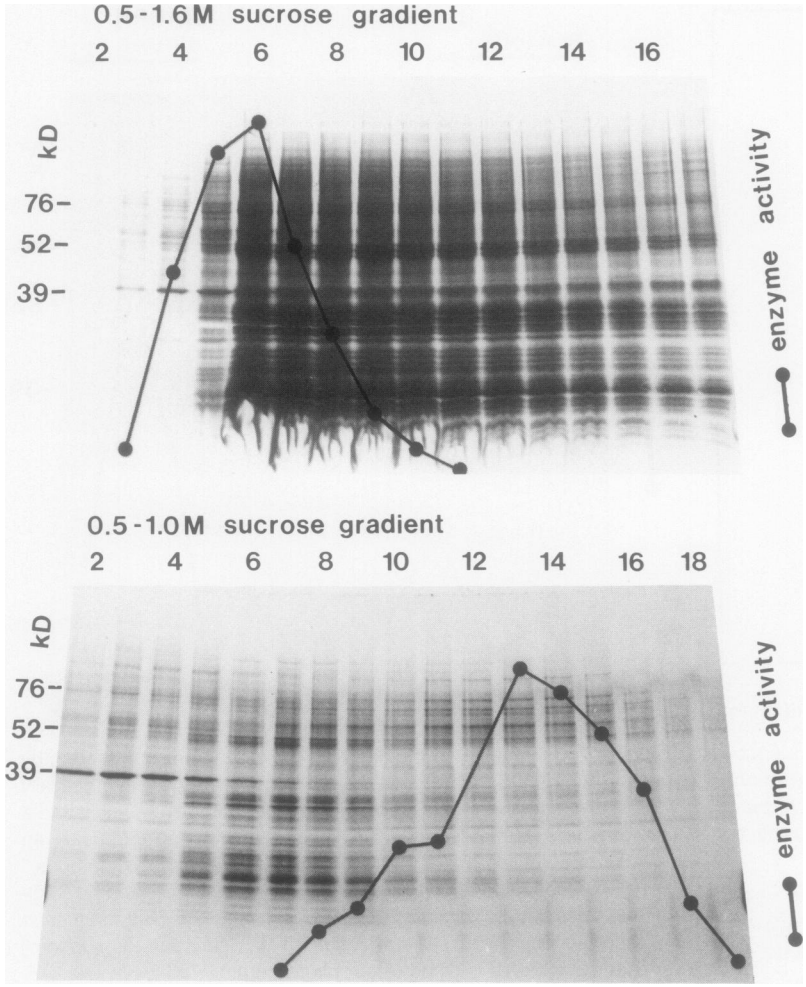

Figure 8. Purification of the dhurrin-synthesizing enzyme system by sucrose gradient centrifugations. Fractions 5 and 6 of the 0.5 to 1.6 M sucrose gradient, containing the whitish protein band were combined and fractionated on a 0.5 to 1. 0 M sucrose gradient. The protein profile of each fraction was analyzed by SDS-PAGE and the gel stained with Coomassie brilliant blue R250. The activity profile of the dhurrin-synthesizing enzyme system, as obtained by analysis of aliquots of each fraction using tyrosine as substrate, is superimposed on the electrophoretograms.

periments with fractions from the 0.5 to 1.6 M sucrose gradient. After combination of different gradient fractions, the corresponding activity clearly exceeded the sum of the activity of the individual fractions (data not shown). This shows that partial dissociation of essential components from the dhurrin-synthesizing enzyme system already occurs at the initial stage of purification. Nevertheless, all the purification methods tested resulted in preparations which were equally active toward tyrosine and *p*-hydroxyphenylacetonitrile.

The dhurrin-synthesizing enzyme system is a highly organized multifunctional enzyme complex bound to microsomal membranes (23). The biosynthesis of dhurrin involves several mono-oxygenases (Fig. 1). Other mono-oxygenases with a similar requirement for NADPH and molecular oxygen contain iron present either as a heme group (2, 15), an iron-sulfur cluster (19), or as another nonheme iron (30). The transfer of electrons from NADPH to the iron atom involves a flavin passage (13). These short electron transport chains of mono-oxygenases are terminated in a substrate-specific component which also mediates the activation of oxygen (29). It is therefore not surprising that all our attempts to solubilize the dhurrin-synthesizing enzyme system from the microsomal membranes by the use of detergents have resulted in a total loss of biosynthetic activity. Our present efforts toward further characterization of the dhurrin-synthesizing enzyme system are aimed at purification of individual components in the complex using spectrometric assays to monitor the purification of individual components and reconstitution experiments to monitor biosynthetic activity.

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