Cytochromes P-450 from Cassava (Manihot esculenta Crantz)
Catalyzing the First Steps in the Biosynthesis of the Cyanogenic
Glucosides Linamarin and Lotaustralin

CLONING, FUNCTIONAL EXPRESSION IN PICHIA PASTORIS, AND SUBSTRATE SPECIFICITY OF THE
ISOLATED RECOMBINANT ENZYMES

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The first committed steps in the biosynthesis of the two cyanogenic glucosides linamarin and lotaustralin in cassava are the conversion of L-valine and L-isoleucine, respectively, to the corresponding oximes. Two full-length cDNA clones that encode cytochromes P-450 catalyzing these reactions have been isolated. The two cassava cytochromes P-450 are 85% identical, share 54% sequence identity to CYP79A1 from sorghum, and have been assigned CYP79D1 and CYP79D2. Functional expression has been achieved using the methylotrophic yeast, Pichia pastoris. The amount of CYP79D1 isolated from 1 liter of P. pastoris culture exceeds the amounts that putatively could be isolated from 22,000 grown-up cassava plants. Each cytochrome P-450 metabolizes L-valine as well as L-isoleucine consistent with the co-occurrence of linamarin and lotaustralin in cassava. CYP79D1 was isolated from P. pastoris. Reconstitution in lipid micelles showed that CYP79D1 has a higher k value with L-valine as substrate than with L-isoleucine, which is consistent with linamarin being the major cyanogenic glucoside in cassava. Both CYP79D1 and CYP79D2 are present in the genome of cassava cultivar MCol22 in agreement with cassava being allotetraploid. CYP79D1 and CYP79D2 are actively transcribed, and production of acyanogenic cassava plants would therefore require down-regulation of both genes.

Cassava (Manihot esculenta Crantz) is the most important tropical root crop (1, 2). Its starchy tuberous roots are the major staple food for several hundred million people. The average annual per capita consumption of cassava is 347 kg in the Democratic Republic of Congo (1994-1996 numbers; Ref. 1). The importance of the cassava plant is increasing for a number of reasons. Cassava produces well on poor soils and can be grown with low input. It is very drought-resistant, and the tubers can be left in the soil for a couple of years. Accordingly, growth of cassava provides an important reserve carbohydrate source to prevent or relieve famine during periods of adverse climate conditions and offers the possibility to obtain a harvest from eroded and otherwise abandoned fields. The crop is propagated vegetatively from stem cuttings. In periods of food shortage, the farmer is therefore not required to save a part of the edible cassava crop for replanting of the fields to obtain next year’s crop. Major deficits of cassava are the low protein content in the tuberous roots, poor storability after harvest, and the high content of cyanogenic glucosides (3). Cassava contains the two cyanogenic glucosides linamarin and lotaustralin in all parts of the plant (4). Upon tissue disruption, the cyanogenic glucosides are degraded with concomitant release of hydrogen cyanide and ketones. The level of cyanide generated from the tubers differs from one variety to the other but ranges between 0.15 and 1.5 g/kg of tuber as calculated on a dry weight basis. Acyanogenic cassava plants are not known (3). Use of cassava products as a staple food thus requires careful processing to remove the cyanide. Inadequate processing may result in chronic cyanide intoxication and tropical ataxic neuropathy (5). Severe cases of cyanide intoxication have been correlated to outbreaks of the paralytic disease konzo (6–8). Typically, processing is labor intensive and time-consuming and results in a simultaneous loss of proteins, vitamins, and minerals (3). Attempts to increase the content of valuable nutrients (e.g. proteins) in cassava tubers by breeding will thus be in vain because the components most likely will be lost during processing. The presence of cyanogenic glucosides thus constitutes a major obstacle for food quality improvement in cassava. Attempts to eliminate the production of cyanogenic glucosides in cassava through traditional breeding have not been successful. An alternative approach would be to use antisense technology, which, however, requires knowledge of the biosynthetic pathway and the enzymes involved and identification of the relevant genes.

The biosynthesis of the L-tyrosine-derived cyanogenic glucoside dhurrin has been elucidated in sorghum (Sorghum bicolor (L.) Moench). The pathway involves an N-hydroxyamino acid, an N,N-dihydroxyamino acid, an E- and Z-oxime, a nitrile, and a cyano hydrin as intermediates (9–11). Studies carried out using microsomes prepared from etiolated cassava seedlings demonstrate the involvement of the same classes of intermediates (12) (Fig. 1). In sorghum, a multifunctional cytochrome P-450 (P-450)1 enzyme (CYP79A1) catalyzes the conversion from amino acid to Z-oxime and a second multifunctional P-450

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1 The abbreviations used are: P-450, cytochrome P-450; TX-114, Triton X-114; Tricine, (N-tris)[hydroxymethyl]methylglycine; val-oxime,
(CYP71E1) catalyzes the conversion from Z-oxime to cyanoxydrin. Final conversion of the cyanoxydrin to dhurrin is achieved by the action of a soluble uridine 5' -diphosphogluco-syl transferase (13). The multifunctional sorghum P-450s have been isolated, cloned, and expressed in *Escherichia coli* (14–18). In addition to cyanogenic glucosides, oximes are also intermediates in the biosyntheses of glucosinolates (mustard oil glucosides), a closely related group of natural products. In glucosinolate biosynthesis, the conversion of amino acids to their corresponding oximes has been reported to involve P-450 (19, 20) and flavin-containing monoxygenases (21) as well as peroxidases (22). Sweeping statements as to the involvement of different types of enzymes in different plants and in dependence of the precursor amino acid used are currently being made (23, 24).

In the present study, we have used a range of inhibitors to document that the initial steps in cyanogenic glucoside synthesis in cassava are catalyzed by P-450. Using PCR strategies, we have cloned two P-450 genes belonging to the CYP79 family. Southern blot analyses did not reveal additional gene copies. Heterologous expression in *Pichia pastoris* documented that both P-450s catalyze the conversion of L-valine as well as L-isoleucine to the corresponding oximes, i.e. the first committed steps in the biosynthesis of linamarin and lotaustralin. The enzyme was isolated from *P. pastoris* and characterized with respect to substrate specificity. The cloning of the two genes provides the necessary tools to construct acyanogenic cassava plants using antisense technology.

**EXPERIMENTAL PROCEDURES**

*Preparation of Cassava Microsomes and Characterization of the Enzyme System—* Cassava (*M. esculenta* Crantz) was grown in a greenhouse at regimes of 14 h of light at 28 °C and 10 h of darkness at 25 °C. Microsomes were prepared from immature folded leaves and petioles of shoot tips by differential centrifugation (12). The resulting microsomal pellet was resuspended in homogenization buffer (250 mM sucrose, 100 mM Tricine, pH 7.9, 50 mM NaCl, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride) and stored at −80 °C.

Immediately before use, microsomes were thawed on ice.

Biosynthetic activity of the cassava microsomes was determined in assay mixtures (total volume, 30 μl) containing 55 μg of microsomal protein, 1 mM NADPH, 0.35 μCi of [U-14C]-valine (246 mCi/mmol, Amersham Pharmacia Biotech), 3.3 mM val-oxime, and 67 mM Tricine, pH 7.9. After incubation (30 min at 30 °C), each assay mixture was extracted with 60 μl of ethyl acetate. The ethyl acetate phase containing the val-oxime was applied to a TLC sheet (Silica Gel 60 F254, Merck) and eluted in dichloromethane/ethanol (85:15, v/v). The maximum content of dimethyl sulfoxide in the reaction mixtures was 6.7% and was shown in control experiments not to inhibit the biosynthetic activity. The direct effect of DPI and tetcyclasis on purified sorghum NADPH-P-450 oxidoreductase was measured (550 nm) as the inhibition of NADPH-P-450 oxidoreductase (0.01 unit) catalyzed NADPH-dependent reduction of cytochrome c (25).

The metabolism of L- and D-amino acids by cassava microsomes was determined in assay mixtures (total volume, 200 μl) containing microsomal protein (20–100 μg), 6 mM substrate (L-Val, D-Val, L-Ile, or D-Ile), 1.5 mM NADPH, 50 mM Tricine, pH 7.9. The reaction mixtures were incubated in silicone septum-covered Eppendorf tubes for 30 min at 30 °C and stopped by injection of 40 μl of 6 N NaOH. The end product cyanide was determined colorimetrically as described previously (26).

**PCR Amplification, Library Screening, and Sequencing of Genes Belonging to the CYP79 Family—** First round PCR amplification reactions (total volume, 20 μl) were carried out in 10 mM Tris-HCl, pH 9.5, 50 mM KCl, 1.5 mM MgCl2 using 0.5 unit of *Taq* DNA polymerase (Amersham Pharmacia Biotech) 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 500 nM of each of the primers 5'-GCCGAATTCCG-GIAAYCCYTICT and 5'-GCCGGATCCG-GDATRTCIGAYTCTG (where R = A or G; Y = C or T; I = inosine; and D = A, G or T), and 10

![CYP79D1 and CYP79D2](image)

**FIG. 1.** Biosynthesis of the cyanogenic glucosides linamarin and lotaustralin in cassava. Linamarin is synthesized from L-valine and lotaustralin from L-isoleucine. Two membrane-bound, multifunctional P-450s catalyze the synthesis of the aglycones that are converted to the cyanogenic glucosides by a soluble uridine 5’-diphosphogluco-syl transferase. In the absence of glucosyl transferase activity, the aglycones spontaneously dissociate and liberate cyanide.
ng of plasmid DNA template. The plasmid DNA template was prepared from a unidirectional plasmid cDNA library in pcDNA2.1 (Invitrogen) made from immature folded leaves and petioles of shoot tips of cassava plants. Thermal cycling parameters were 95 °C for 2 min, 35 × (95 °C for 1 s, 50 °C for 1 s, and 72 °C for 1 s), followed by a final 72 °C for 5 min. A part of the bands of the expected size (210 base pairs) was stained out with a Pasteur pipette and used for second round PCR amplifications in 50 μl of the same reaction mixture as above using 95 °C for 2 min, 20 × (95 °C for 5 s, 50 °C for 5 s, and 72 °C for 45 s), followed by a final 72 °C for 5 min. The product was sequenced with the Thermo Sequenase radiolabeled terminator reaction mixture as above using 95 °C for 2 min, 20

stream of the start ATG codon. pPICZc was restricted with

BI and

CYP79D2. The adapter reconstructs the first 18 base pairs of

Xho

N-terminal Sequencing—CYP79D1 was subjected to SDS-PAGE and the protein transferred to ProBlott membranes (Applied Biosystems, Foster City, CA) as described previously (33). The Coomassie Brilliant Blue-stained proteins were excised from the membrane and subjected to sequencing on an Applied Biosystems model 470A sequenator equipped with an on-line model 120A phenylthiohydantoin amino acid analyzer. Asn glycosylation was detected as the lack of an Asn signal in the position Eddman degraded Edman sequencing profile.

Catalytic Activity of CYP79D1 and CYP79D2—Isolated, recombinant CYP79D1 was reconstituted, and its catalytic activity was determined in vitro using reaction mixtures (total volume, 30 μl) containing 2.5 pmol of CYP79D1, 0.05 unit of NADPH-P-450 oxidoreductase (25), 10.6 mM 1-t,4-dioleyl phosphatidylcholine, 0.35 μCi of U-14C-labeled l-α-amino acid (l-Val, l-Leu, l-Tyr or l-Phe; Amersham Pharmacia Biotech), 1 μM NADPH, 0.1 mM NaCl, and 20 mM KCl, pH 7.9. In assays containing [14C]-valine or [14C]-isooleucine, different amounts of unlabeled l- and l-α-amino acids (0–6 mm) were added. After incubation (10 min at 30 °C), the products formed were extracted into ethyl acetate (60 μl) and separated on TLC sheets using n-pentane/diethyl ether (50:50, v/v) or toluene/ethyl acetate (5:1, v/v) as eluents for aliphatic compounds and aromatic compounds, respectively. [14C]-Labeled oximes were visualized by autoradiography using a STORM 840 PhosphorImager (Molecular Dynamics). The activity of CYP79D1 was also measured in the presence of the inhibitors tetcyclaz, ABT and DPI, under the same conditions as described in the previous section.

For in vivo activity assays, P. pastoris cells (200 μl) were pelleted and resuspended in 100 μl of 50 mM Tricine, pH 7.9, and 0.35 μCi of [U-14C]-valine or [14C]-isooleucine. After incubation (30 min at 30 °C), the products formed were extracted with ethyl acetate, and the products formed were analyzed as described above.

Additional Analytical Procedures—SDS-PAGE was performed using high Tris linear 8–25% gradient gels (34). Total P-450 was quantified by carbon monoxide difference spectroscopy on a SLM Aminco DW-2000 TM spectrophotometer (Spectronic Instruments, Rochester, NY) using a molar extinction coefficient of 91 mm2 M−1 cm−1 for the adduct between reduced P-450 and carbon monoxide (35). Substrate-binding spectra
were recorded according to the method of Jefcoate (36) in 50 mM KP, pH 7.9, 50 mM NaCl.

RESULTS

P-450-dependent Biosynthetic Activity in Young Cassava Leaves—Microsomes prepared from immature folded leaves and petioles of shoot tips from 3–6-month-old cassava plants converted L-valine to val-oxime (Fig. 2A, lane 2). Microsomes prepared from mature cassava leaves showed barely detectable activity levels. The conversion of amino acids to oxime compounds has been suggested to involve three different enzyme systems: P-450s (19, 20), flavin-containing monoxygenases (21), and peroxidases (22). To determine which enzyme was involved in cassava, ABT and tetcyclasis were tested as inhibitors of P-450 enzymes, and DPI was tested as an inhibitor of the putative flavin-containing monooxygenase. A major argument for the involvement of a flavin-containing monooxygenase has been the observed inhibitory effect caused by the flavin protein inhibitor DPI (21). The production of val-oxime by the cassava microsomes was indeed completely inhibited by DPI (Fig. 2A, lanes 7 and 8). However, as an inhibitor of flavin-containing enzymes DPI may also inhibit the P-450-NADPH oxidoreductase. The inhibitory effect of DPI on NADPH-P-450 oxidoreductase was measured in a cytochrome c reductase assay (25) using NADPH-P-450 oxidoreductase purified from sorghum. The NADPH-P-450 oxidoreductase was found to be completely inhibited (data not shown), suggesting that DPI interferes with the L-valine to val-oxime conversion by inhibiting NADPH-P-450 oxidoreductase. The conversion of L-valine to val-oxime was inhibited by tetcyclasis, which strongly argues for the involvement of a P-450 enzyme (Fig. 2A, lanes 3 and 4). The NADPH-P-450 oxidoreductase was fully active in the presence of tetcyclasis (data not shown). ABT did not inhibit the reaction (Fig. 2A, lanes 5 and 6). ABT is not a universal P-450 inhibitor (37, 38), and the lack of inhibition is therefore not necessarily in conflict with a P-450 catalyzed reaction. The L-valine to val-oxime conversion is dependent on NADPH (Fig. 2A, lane 1) as required for a P-450 catalyzed reaction. The overall conclusion from the inhibitor studies is that the L-valine to val-oxime conversion in cassava is a P-450-catalyzed reaction.

CYP79D1 and CYP79D2—Based on the assumption that the P-450 catalyzing conversion of L-valine to val-oxime belongs to the CYP79 family, degenerate primers were designed toward areas showing sequence conservation in CYP79A1 (sorghum) (15), CYP79B1 (Sinapis alba) (20), and CYP79B2 (Arabidopsis thaliana) (20). Domains putatively involved in substrate recognition (39) were excluded for primer designation, because none of the known CYP79s utilize valine or isoleucine as substrate. PCR was performed with the degenerate primers on plasmid DNA from a cDNA library prepared from immature folded leaves and petioles from shoot tips of cassava plants. PCR using the conserved areas shown by arrows in Fig. 3 was successful and provided a cassava sequence with high sequence similarity to the CYP79 family. Using this probe, two equally abundant full-length clones were isolated from the cassava cDNA library. The clones have open reading frames encoding P-450s of 61.2 and 61.3 kDa. These P-450s have been assigned CYP79D1 and CYP79D2 as the first two members of a new CYP79 subfamily (Dr. Nelson, P-450 Nomenclature Committee; Ref. 40). The two cassava P-450s are 85% identical and both share 54% identity to CYP79A1. P-450s showing more than 40% but less than 55% sequence identity to the amino acid level are grouped in the same family but in different subfamilies (41). Fig. 3 shows an alignment of CYP79D1 and CYP97D2 with CYP79A1, the only member of the CYP79 family for which a function has been established, and with CYP79B1 and CYP79B2, which possibly are involved in glucosinolate biosynthesis.

The heme-binding motif in CYP79D1 and CYP79D2 is TFSTGRRCGVAC (Fig. 3, residues 470–480 for CYP79D1) and contains three amino acid substitutions compared with the consensus sequence PFGXGRRCXG for A-type P-450s (41). The substitutions underlined are also found in CYP79A1, whereas the initial Thr in the CYP79D1 and CYP79D2 heme-binding motif is a Ser in CYP79A1, CYP79B1, and CYP79B2. Thus, the previously proposed existence of a heme binding sequence domain unique to the CYP79 family (20) is contradicted. The other unique sequence domain PERH (Fig. 3, residues 450–453 for CYP79D1), where His is proposed to be specific for the CYP79 family (20), is also found in CYP79D1 and CYP79D2. The central region of helix I is suggested to be involved in the formation of the substrate binding pocket (42) and contains the consensus sequence (A/G)X(3,4)(I/R)X(3,4)(D/E)X(I/T/T/S) (41). A replacement in CYP79A1 (Fig. 3, corresponding to residues 342–343 in CYP79D1) of the consensus Thr(Thr/Ser) with Asp-Pro was previously interpreted to be involved in defining tyrosine binding (15). However, NP residues are also present in CYP79D1 and CYP79D2, as well as in CYP79B1 and CYP79B2, suggesting that these residues are typical to the CYP79 family independent of the substrate used.

Copy Number of CYP79D1 and CYP79D2—To determine the copy number of CYP79D1 and CYP79D2, a Southern blot on
genomic DNA from the cassava cultivar MCol22 was performed. The blot was hybridized with CYP79D1 and CYP79D2 cDNAs. The two probes hybridized to different bands on the Southern blot (Fig. 4), demonstrating that both genes are present in the MCol22 genome. The high similarity between the genes results in weak cross-hybridization, e.g., in Fig. 4A (lane BglII), where a weak band at the size corresponding to CYP79D2 is observed. Low stringency washing (0.5× SSC, 0.1% SDS at 55 °C) did not reveal additional copies of the CYP79D genes (data not shown).

Functional Expression in P. pastoris—Expression of CYP79D1 and CYP79D2 in E. coli using constructs and conditions similar to those tested to achieve expression of sorghum CYP79A1 (16) was unsuccessful. Similarly, it has been reported that CYP79B1 is not expressed in E. coli (20). The methylotropic yeast P. pastoris was explored as an alternative expression system. To optimize expression conditions, the 5'- and 3'-untranslated regions were removed from the cassava genes before insertion into the expression vector. The start ATG was positioned exactly as the start ATG of the highly expressed AOX1 P. pastoris gene to obtain an optimal translation initiation context.

CYP79D1 and CYP79D2 were functionally expressed in P. pastoris as evidenced by the ability of recombinant yeast cells to convert l-valine to val-oxime (Fig. 5, lanes 2 and 3). No conversion took place using P. pastoris cells transformed with the vector only (Fig. 5, lane 1). The metabolic activity was measured in intact cells, demonstrating that the endogenous P. pastoris reductase system is able to support electron donation to these plant P-450s. SDS-PAGE of microsomes prepared from cells actively converting l-valine to val-oxime showed the presence of an additional polypeptide band migrating correspond-
of microsomal CYP79D1 after 90 h of induction was 50% of that obtained after 24 h (data not shown).

**Purification of Recombinant CYP79D1**—Recombinant CYP79D1 was isolated from *P. pastoris* microsomes by initial solubilization in TX-114 followed by TX-114 temperature-induced phase partitioning and centrifugation at room temperature to recover CYP79D1 in the reddish TX-114-rich upper phase. Re-extraction of the TX-114-poor phase to recover residual CYP79D1 provided quantitative extraction as estimated by CO difference spectroscopy (Fig. 6A, lane 3). The rich phases were combined and diluted to 0.2% TX-114 before application to a column of DEAE mounted in series with a column of Reactive Red 120 agarose. The DEAE column binds the majority of microsomal protein. CYP79D1 binds weakly to DEAE and was transferred to the red column upon washing with 10% glycerol. CYP79D1 was eluted as a nearly homogenous protein from the red column (Fig. 6A, lane 4) and was isolated on a column of Reactive Yellow 3A agarose (Fig. 6A, lane 5). The isolated CYP79D1 migrated with a molecular mass of 62 kDa. The overall yield of the isolation procedure was 17%, i.e. 1 nmol of CYP79D1 was obtained from each 260 ml of culture.

**CYP79D1 Expressed in P. pastoris Is Glycosylated**—The fractions that produced CO spectra and contained CYP79D1 activity always produced two distinct closely migrating polypeptide bands upon SDS-PAGE (Fig. 6A, lane 5). N-terminal amino acid sequencing identified both bands as derived from CYP79D1 (Fig. 6B). The transient methionine was removed by the yeast processing system. Sequence of the first 15 residues of the upper band (D1-Gly, Gly) demonstrated glycosylation of both asparagines present, whereas the lower band (D1-Gly) only was glycosylated at the first asparagine. The glycosylation pattern explains the presence of two bands. Glycosylation at the N-terminal part of CYP79D1 is in agreement with the localization of the N-terminal in the lumen of the endoplasmatic reticulum accessible for the glycosylation machinery. It is unknown whether native CYP79D1 is glycosylated in cassava. However, CYP79D1 purified from sorghum seedlings was not glycosylated as documented by amino acid sequencing of the N-terminal fragment (15), and only few reports exist of microsomal P-450 glycosylation (43, 44). The observed glycosylation of recombinant CYP79D1 upon expression in *P. pastoris* is thought to reflect expression in a yeast system.

**Spectral Properties of Recombinant CYP79D1**—Isolated CYP79D1 consistently produced an absorption maximum at 448 nm when subjected to CO difference spectroscopy (Fig. 7A). No maximum was observed at 420 nm using either isolated or crude fractions. This demonstrates that CYP79D1 is a fairly stable protein. Yeast cytochromes may interfere with the spectroscopy of crude extracts and hide a minor 420 nm peak, and *P. pastoris* cytochrome oxidase had previously been reported to prevent P-450 spectroscopy (45). In the present study, the expression level of CYP79D1 was high, and the CO difference spectrum produced by cytochrome oxidase (maximum at 430 nm, minimum at 445) (46) is visible as a shoulder on the 450 nm peak. The *P. pastoris* cytochrome oxidase binds to the DEAE column and accordingly was removed during P-450 isolation. Upon culturing *P. pastoris* for extended periods (90 h), the content of cytochrome oxidase decreased permitting readily detection of lower amounts of P-450 in microsomes. Finally, interfering cytochrome oxidase could be removed from P-450 by TX-114 phase partitioning performed in borate buffer (data not shown). Upon phase partitioning in borate, the P-450s partitioned to the TX-114-poor phase (47), whereas *P. pastoris* cytochrome oxidase partitioned to the rich phase. Isolated CYP79D1 formed a type I substrate binding spectrum in the
Substrate selectivity of CYP79D1—CYP79D1 was reconstituted with sorghum NADPH-P-450 oxidoreductase in the presence of 8 mM L-valine.

Substrate Specificity of CYP79D1—CYP79D1 was reconstituted with high spin to low spin state upon substrate binding.

Cyanogenic Glucoside Biosynthesis in Cassava

To examine the effect of inhibitors on isolated CYP79D1, reconstitutions were performed in the presence of tetcyclasis, ABT, and DPI (Fig. 2A) using the same conditions as for cassava microsomes. The same pattern as in cassava microsomes is observed using isolated CYP79D1. CYP79D1 is inhibited by tetcyclasis but not by ABT. Similar to the situation in cassava microsomes, DPI completely inhibits the val-oxime formation by inhibiting the NADPH-P-450 oxidoreductase.

The ability of cassava microsomes and reconstituted CYP79D1 to metabolize the d-isomers of valine and isoleucine was examined. When cassava microsomes were used, cyanide was produced with L-valine and L-isoleucine as substrates (Fig. 1), whereas no metabolism was observed using d-valine and d-isoleucine (Fig. 9A). A higher conversion rate is observed using L-valine compared with L-isoleucine similar to the data obtained using microsomes prepared from etiolated cassava seedlings (12). Isolated CYP79D1 produces 14C-labeled val-oxime from 14C-L-valine (Fig. 9B, lane 1). When the specific activity of the 14C-L-valine substrate is reduced 120 times by addition of unlabeled L-valine, a corresponding reduction of the amount of 14C-labeled val-oxime formed is observed (Fig. 9B, lane 2). However, addition of unlabeled d-valine to the incubation mixture does not result in a corresponding reduction in the amount of 14C-labeled val-oxime formed (Fig. 9B, lane 3). Thus, neither the cassava microsomes nor isolated CYP79D1 metabolize d-valine. The lack of competition of d-valine with L-valine indicates that d-valine does not bind with high affinity to the active site of CYP79D1. Similar results were obtained with 14C-L-isoleucine, L-isoleucine, and d-isoleucine (data not shown).

To further investigate the kinetics of L-valine and L-isoleucine metabolism by isolated CYP79D1, assays were performed over a broad range of substrate concentrations (Fig. 9C). Eadie-Hofstee plots (Fig. 9D) of the experiment shown provided the following parameters: $K_m$ values of 1.2–2.2 mM and $k_c$ values of 4.3–9.7 min$^{-1}$ for L-valine metabolism and $K_m$ values of 1.3–1.7 mM and $k_c$ values of 2.3–6.4 min$^{-1}$ for L-isoleucine metabolism. Under saturating substrate conditions CYP79D1 has a higher conversion rate using L-valine as substrate. The conversion rate of L-isoleucine is approximately 60% of that observed for L-valine. This is consistent with higher accumulation of linamarin compared with lotaustralin in vivo in cassava (4).

Characterization of CYP79D2—Assays based on intact cells as well as microsomes of P. pastoris demonstrated that CYP79D2 metabolized L-valine (Fig. 5, lane 3) and L-isoleucine (data not shown) to the corresponding oximes. Thus, both CYP79D1 and CYP79D2 are able to catalyze the first part of the biosynthesis of linamarin and lotaustralin in cassava.

P. pastoris cultures that produced CYP79D1 and CYP79D2 (28 h of induction) with similar rate of L-valine to val-oxime conversion afforded very different yields of P-450 in microsomal preparations. Whereas recovery of P-450 in microsomes of CYP79D1 was 15–30 nmol/liter culture, the amount of CYP79D2 in microsomes was too low to be detected because of cytochrome oxidase interference with the CO spectrum. CYP79D2 was only detectable after removal of the cytochrome oxidase by TX-114 phase partitioning in borate (47) (data not shown). The glass bead method used for breaking the P. pastoris cells is a harsh procedure and may have denatured CYP79D2.
The first committed steps in the biosynthesis of linamarin and lotaustralin in cassava are the conversion of L-valine and L-isoleucine, respectively, to the corresponding oximes. Two full-length cDNA clones were isolated that encode P-450s catalyzing these reactions. The two P-450s showed 54% sequence identity to CYP79A1 from sorghum and were assigned CYP79D1 and CYP79D2 as the first two members of a new CYP79 subfamily. A member of the CYP79 family has been obtained from *S. alba* (20) and additional members are derived from the *A. thaliana* genome and expressed sequence tag sequencing programs. Unambiguous assignment of these CYP79s as amino acid to oxime converting enzymes in glucosinolate biosynthesis has not been possible because heterologous expression of these genes, as well as of CYP79D1 and CYP79D2 from cassava, in *E. coli* has so far not been successful (20). It thus remains to be documented whether all members of the CYP79 family catalyze amino acid to oxime conversions and are functionally conserved.

In the present study, functional expression of CYP79D1 and CYP79D2 from cassava was achieved in the methylotrophic yeast, *P. pastoris* (48, 49). A number of soluble proteins have been expressed in *P. pastoris* with expression levels in the range of g/liter (e.g. 50, 51). In contrast, reports on membrane protein expression in *P. pastoris* are few. Only two P-450s from spiny dogfish shark and spiny lobster have previously been expressed (45, 52) as evidenced by a new expected enzyme activity in the transformed cells. However, these recombinant P-450 proteins were neither characterized nor isolated. In *E. coli*, expression usually requires different types of bovine 17a modifications (53). CYP79D1 and CYP79D2 were functionally expressed in *P. pastoris* using the native sequences. The expression level was in the range of 15–30 nmol CYP79D1/liter of culture as measured by CYP79D1 recovered in the microsomal pellet. The actual amount of CYP79D1 in the intact cells is probably much higher because the procedure for cell disintegration is not efficient. The expression level is 125 pmol/mg microsomal protein, which is similar to the average levels obtained in *Saccharomyces cerevisiae* (54). The translation initiation context of the two cassava genes were optimized, which may explain the more than six times higher levels of expression obtained compared with the spiny dogfish shark and spiny lobster P-450s.

Recombinant CYP79D1 produces a typical CO spectrum and a type I substrate-binding spectrum in the presence of L-valine, indicating that the *P. pastoris* expression system produces a correctly folded P-450 protein. The N-glycosylations found in the N-terminal part of the protein documents that *P. pastoris* inserted the protein in the endoplasmatic reticulum with the correct orientation. CYP79D1 is not expected to be glycosylated in cassava because previous reports of P-450 glycosylations are scarce (43, 44). The relative mobility of recombinant CYP79D1 on SDS-PAGE corresponds well to the mass predicted from the cDNA clone, and thus CYP79D1 is expected to have a simple glycosylation structure. Hyperglycosylations is generally observed as a disadvantage of *S. cerevisiae* expression systems. In contrast *P. pastoris* adds smaller sized oligosaccharides to the protein (8–14 mannos residues/side chain; Ref. 55).

As a yeast, *P. pastoris* contains endogenous P-450 proteins (40). However, under the experimental conditions used, these must be expressed in low amounts, because endogenous P-450s were never detectable by CO spectroscopy. This property renders *P. pastoris* suited for detection of heterologous P-450 expression by spectroscopy alone. Of greater concern is the cytochrome oxidase protein that forms a spectrum in the presence of CO, which interferes with P-450 spectroscopy. However, the
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cytchrome oxidase can easily be removed from the P-450s by Triton X-114 phase partitioning in borate buffer or by DEAE chromatography. In conclusion, we consider P. pastoris a very suitable organism for P-450 expression.

The conversion of the parent amino acid to the corresponding Z-oxime is the rate-limiting step in the biosynthetic pathway in sorghum (56) and cassava (12). In cassava, microsomes as well as CYP79D1 exhibited a higher \( V_{\text{max}} \) value with L-valine compared with L-isoleucine. This is consistent with linamarin being the major cyanogenic glucoside in cassava (ratio linamarin/lotaustralin 93:7; Ref. 4). The rate of L-isoleucine metabolism is higher than expected, indicating that the availability of the parent amino acids may be a limiting factor in vivo. In line with this suggestion, microsomes prepared from cassava cultivars known to contain low amounts of cyanogenic glucosides show higher biosynthetic activity compared with plants known to be highly cyanogenic (57, 58). Linamarin and lotaustralin almost always co-occur in plants (11). This is consistent with the findings of the present study, demonstrating that CYP79D1 and CYP79D2 each are able to metabolize L-valine as well as L-isoleucine. In white clover (Trifolium repens L.), lotaustralin predominates linamarin (59) and microsomes prepared from this plant preferentially metabolizes l-isoleucine (60). The substrate preference of the first committed enzyme in cyanogenic glucosides biosynthesis thus appears to be a major determinant of the ratio between accumulated cyanogenic glucosides. As previously discussed in Koch et al. (12), the kinetic constants observed for valine metabolism in cassava microsomes (\( K_m = 1.9 \text{ mM}, \ V_{\text{max}} = 19 \text{ mmol/h/mg microsomal protein} \)) was too poor to explain the in vivo level of cyanogenic glucosides observed. It was concluded that the enzyme system recovered in the cassava microsomes was labile (12). Similarly, isolated and reconstituted CYP79D1 exhibits high \( K_m \) values above the expected in vivo substrate concentrations. Unless the free amino acids used for cyanogenic glucoside synthesis are compartmentalized, it may be speculated that it would not be advantageous for a plant to have a highly effective enzyme system depleting the general pool of free amino acids in the plant. However, the turnover rates of reconstituted CYP79D1 are also low. In this study, CYP79D1 has been reconstituted with NADPH-P-450 oxoreductase isolated from S. bicolor. The electron transfer process might have been more effective using native cassava NADPH-P-450 oxoreductase. The reconstituted, recombinant CYP79D1 enzyme isolated in the presence of detergents represents a potentially more labile system than cassava microsomes (12). It can be expected that the actual in vivo synthesis rates are higher.

The CYP79D1 and CYP79D2 clones were obtained using PCR strategies based on sequence comparisons with other members of the CYP79 family. A parallel biochemical approach to isolate the proteins was unsuccessful. Young etiolated seedlings are biosynthetically active (12). However, residual parts of the seed coat remain firmly attached to the cotyledons and contain a potent inhibitor of the enzyme system. These remnants need to be removed manually from each seedling precluding work-up of a large number of seedlings. The biosynthetic activity detected in the root phelloderm is very low and difficult to recover because of large amounts of starch (57). Immature folded leaves and petioles from shoot tips of cassava seedlings turned out to be the most convenient tissue to work with because they possessed as much activity as etiolated seedlings. In contrast, biosynthetic activity is barely detected in mature leaves. The amount of CYP79D1 in 1 liter of P. pastoris culture contains roughly to the amount in 0.4 kg of immature folded leaves and petioles of cassava. Purification from plant material generally results in a very low yield because of pigments and co-occurring phenolic compounds. Thus, CYP79A1 was purified from E. coli in 15% yield compared with a 1% yield from plant material (14, 16). Considering the different yields and recognizing that one cassava plant provides approximately 0.3 g of the immature folded leaves and petioles, a minimum number of 22,000 grown-up cassava plants would be necessary to provide the same amount of purified CYP79D1 as obtained from 1 liter of P. pastoris culture.

Both CYP79D1 and CYP79D2 are present in the genome of cassava cultivar MC022. Southern blot analysis did not reveal additional gene copies. Preliminary data show the presence of transcripts of both genes in the cassava plant, e.g. in the young cassava petioles. This demonstrates that both genes could be responsible for cyanogenic glucoside production in cassava and should be targeted by the antisense constructs used in attempts to block or down-regulate cyanogenic glucoside synthesis in cassava. The presence of two homologous genes in the cassava genome each encoding a P-450 able to catalyze the initial steps in linamarin and lotaustralin biosynthesis is in agreement with cassava being allotetraploid. Recently, a genetic cassava map has been developed (61), and mapping of the CYP79D1 and CYP79D2 genes is in progress.

The types of intermediates formed in cyanogenic glucoside biosynthesis are highly unusual and not known to take part in other metabolic pathways in the same plants. Thus the plant is not expected to utilize these intermediates for other purposes, nor are the intermediates expected to be generated from other biosynthetic pathways. Down-regulation of the expression of the CYP79D1 and CYP79D2 genes by antisense techniques to obtain a cyanogenic cassava plants would therefore appear possible without pleiotropic metabolic effects. Work along these lines is now in progress, taking advantage of the recently developed procedures for cassava transformation (62).

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