Molecular Cloning of the Full-length cDNA of (S)-Hydroxynitrile Lyase from Hevea brasiliensis

FUNCTIONAL EXPRESSION IN ESCHERICHIA COLI AND SACCHAROMYCES CERESVIAE AND IDENTIFICATION OF AN ACTIVE SITE RESIDUE*

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The full-length cDNA of (S)-hydroxynitrile lyase (Hnl) from leaves of Hevea brasiliensis (tropical rubber tree) was cloned by an immunoscreening and sequenced. Hnl from H. brasiliensis is involved in the biodegradation of cyanogenic glycosides and also catalyzes the stereospecific synthesis of aliphatic, aromatic, and heterocyclic cyanohydrins, which are important as precursors for pharmaceutical compounds. The open reading frame identified in a 1.1-kilobase cDNA fragment codes for a protein of 257 amino acids with a predicted molecular mass of 29.2 kDa. The derived protein sequence is closely related to the (S)-hydroxynitrile lyase from Manihot esculenta (Cassava) and also shows significant homology to two proteins of Oryza sativa with as yet unknown enzymatic function. The H. brasiliensis protein was expressed in Escherichia coli and Saccharomyces cerevisiae and isolated in an active form from the respective soluble fractions. Replacement of cysteine 81 by serine drastically reduced activity of the heterologous enzyme, suggesting a role for this amino acid residue in the catalytic action of Hnl.

Hydroxynitrile lyases (Hnls)† are involved in a process termed “cyanogenesis” and catalyze the final step in the biodegradation pathway of cyanogenic glycosides in plant cells. The degradation starts with the hydrolysis of cyanogenic glycosides by β-glicosidases (1) to form the aglycons, α-hydroxynitriles, or cyanohydrins. The unstable aglycons are then cleaved into HCN and the corresponding carbonyl component by Hnl. Hydroxynitrile lyases were first described by Rosenthaler (2) at the beginning of this century. HCN released by Hnl can either serve as a repellent factor to predators (3) or as a susceptibility component of plants to fungal attack (4) when produced at high local concentrations. A potential physiological role for cyanogenic glycosides as storage compounds for nitrogen has also been suggested (5). HCN released from cyanohydrins can be fixed by β-cyanoalanine synthase and then used for amino acid biosynthesis in plants. α-Hydroxynitrile lyases have attracted the interest of several laboratories, as they can be used as biocatalysts for the production of enantiomerically pure stereoisomers of cyanohydrins (6). Hnls catalyze the stereospecific retrodiation of a great number of aliphatic, aromatic, and heterocyclic cyanohydrins from HCN and aldehydes or ketones, respectively. Chiral cyanohydrins are the starting material for the synthesis of many important pharmacologically active compounds (6).

α-Hydroxynitrile lyases have been isolated and partially characterized from a variety of species and assigned to two classes, depending on their properties. The first class of Hnls, isolated from Rosaceae, are single chain glycoproteins with up to 30% carbohydrate content and a molecular mass between 50 and 80 kDa. These enzymes are co-factor-dependent, with FAD as the prosthetic group, and accept (R)(±)-mandelonitrile as their substrate. (R)-Hnls from several species of Rosaceae appear to be related to flavoproteins, such as alcohol or glucose dehydrogenases, or glucose oxidases (7). A cysteine residue has been shown to be required for catalytic activity (8, 9), whereas the function of the FAD prosthetic group is still a matter of debate. These enzymes are present in relatively high concentrations in seed tissues and require only 5–10-fold purification to reach homogeneity.

The second class of Hnls are co-factor-independent with a subunit molecular mass of 28–42 kDa. Active enzymes of this type have been isolated from different families (Oliaceae, Gramineae, Linaceae, Euphorbiaceae), contain up to 9% carbohydrate, and adopt homo- or heterodimemic conformations in vivo. Class II Hnls accept (S)-mandelonitrile, 4-OH-(S)-mandelonitrile, acetone cyanhydrin, or (R)-2-butanone cyanhydrin as substrates (see Table 1) and require more than 100-fold purification to reach homogeneity. (S)-Hydroxynitrile lyase from Sorghum bicolor (Gramineae) is immunologically related to carboxypeptidases (10) and shows significant homology to various carboxypeptidases at the amino acid level. A potential catalytic triad has been identified that includes a serine, an aspartate, and a histidine (11).

α-Hydroxynitrile lyase from Hevea brasiliensis (tropical rubber tree) is a class II enzyme and has been purified to homogeneity by Schall et al. (2). The subunit molecular mass of the unglycosylated enzyme is 30 kDa, and the enzyme activity is co-factor-independent. In low salt buffer solutions the enzyme is active as a homodimer. In contrast to other hydroxynitrile

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‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U40402.

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The abbreviations used are: Hnl, (S)-hydroxynitrile lyase; PCR, polymerase chain reaction.

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‡ M. Schall, H. Griengl, and M. Hayn, manuscript in preparation.
lyases, Hnl from *H. brasiliensis* has specificity for both aliphatic and aromatic (S)-cyanohydrins (11). Here we report on the molecular cloning and sequence of a full-length cDNA of Hnl from *Hevea brasiliensis*. The cDNA was expressed in *E. coli* and Saccharomyces cerevisiae, and functional enzyme was produced and isolated from both systems. Using inhibitors and in vitro mutagenesis, we show that amino acid Cys81 is required for enzymatic phase.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids—** *E. coli* XL1-Blue (endA1, hsdR17 (rK-, mK+), supE44, thi-1, lambda recA1, gyrA96, relA1, lacI(q), F′proAB, lacZΔM15, Tn10TetR) was used for plasmid amplification and protein expression. *E. coli* TOP10 (mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZAM15, ΔlacX74, deyR, recA1, araD139ΔscarA1, leu2-3, leu2-3, 35). 1 ml of the mixture was assayed for activity. For inhibition studies (inhibitors used are listed in Table II) 1 ml of enzyme extract in assay buffer was incubated with 10 mU of mixture for 30 min at room temperature. 25–50 μl of the mixture was used for kinetic assay.

**Preparation of Rabbit Polyclonal Anti-Hnl Antiserum—** *H. brasiliensis* was purified to homogeneity from *Hevea brasiliensis* leaves and used for induction of anti-Hnl antibodies in rabbit applying standard immunization techniques. Titer and monospecificity of obtained anti-Hnl antiserum were tested by Western blotting techniques, using protein extracts from *Hevea brasiliensis*.

**DNA and RNA Manipulations—** DNA manipulations, plasmid transformation into *E. coli*, and retrieval of DNA from *E. coli* were performed following standard procedures (14). Plant genomic DNA was isolated by the method of Rogers and Reichardt as described in Ausubel et al. (14). Total RNA was prepared from fresh leaves of *H. brasiliensis* (provided by the Botanical Garden of the Karl-Franzens Universität, Graz) following a guanidinium thiocyanate cleavage extraction method (15). Poly(A) RNA was enriched on oligo-dT cellulose (16) using the mRNA Separator Kit (Clontech Laboratories Inc., Palo Alto, CA). RNA was separated under denaturing conditions on 10% formaldehyde agarose gels in a MOPS buffer system (20 mm 3-(N-morpholino)propanesulfonic acid, 1 mm EDTA; pH 7.4). Hybridization of RNA or DNA transferred to nylon filters was performed as described (14) using a nonradioactive digoxigenin-probe labeling system (Boehringer Mannheim). An oriented cDNA library was constructed by employing a Uni-ZAP XR phage vector and Gigapack Gold II packaging system, according to the supplier’s instructions (Stratagene Cloning Systems, La Jolla, CA).

**Enzyme Assay—** Enzyme activity was determined by following the formation of benzaldehyde from 3.8 mM racemic mandelonitrile in assay buffer (50 mM sodium citrate buffer, pH 5.0). The increase of benzaldehyde absorbance at 280 nm was monitored at 25°C over 5 min, which was in the linear range of the assay. For inhibition studies (inhibitors used are listed in Table II) 1 ml of enzyme extract in assay buffer was incubated with 10 mU of inhibitor for 30 min at room temperature.

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**Immunoscreening of the cDNA Library—** Phage from the library were plated at a density of approximately 10^6 plaque-forming units on NZY plates (0.5% NaCl, 0.2% MgSO_4·H_2O, 0.5% yeast extract, 1% NZ amine, 2% agar, 0.150 mm) using *E. coli* XLI-Blue host cells (preculture grown to A_600 = 1.0). After cultivating phage for 8–10 h, plates were overlaid with a nylon filter soaked in 10 mm isopropyl-1-thio-β-D-galactopyranoside, and expression from the lac promoter was induced by incubation for 3 h. Filters were removed from the plates, blocked, and incubated desalted on Econo-Pac 10 DG columns (Bio-Rad) and stored at −20°C.

**Assay of Hnl Activity—** Enzyme activity was determined by following the formation of benzaldehyde from 3.8 mM racemic mandelonitrile in assay buffer (50 mM sodium citrate buffer, pH 5.0). The increase of benzaldehyde absorbance at 280 nm was monitored at 25°C over 5 min, which was in the linear range of the assay. For inhibition studies (inhibitors used are listed in Table II) 1 ml of enzyme extract in assay buffer was incubated with 10 mU of inhibitor for 30 min at room temperature. 25–50 μl of the mixture was used for assay.

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3 N. Klempier, personal communication.
with anti-Hnl primary antibody and anti-rabbit secondary antibody following the protocol of the Western Light Chemoluminescent Detection System (Tropix Inc., Bedford, MA). Anti-Hnl antiserum was diluted in 1/3 PBS (0.65 M NaPO₄, 0.68 M NaCl, pH 7.4) containing 0.1% Tween 20 and 0.1% I-Block (Tropix Inc.). Specifically bound antibodies were visualized using alkaline phosphatase and the chromogenic substrate nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, 4-toluindine salt (Boehringer Mannheim). Positive phage clones were converted to plasmid derivatives of pBluescript SK by in vivo excision (Stratagene Cloning Systems, La Jolla, CA). Heterologous proteins were characterized by SDS-polyacrylamide gel electrophoresis and Western blot analysis of E. coli extracts, leading to the identification of clone pHNL-100.

Sequence Analysis—DNA-sequencing was performed by the chain termination method (17) using an ABI 373A automated sequencer and the Dye Deoxy Terminator Sequencing Kit (Applied Biosystems Inc.). The sequence of N-terminal and internal peptide fragments generated by proteolytic cleavage of purified Hnl with immobilized Lys-C was determined at a commercial protein sequencing facility (TopLab GmbH, FRG).

Comparison to nucleic acid sequence data bases (GenBank™ release 84.0 and EMBL release 39.0, and releases since) and protein data bases (Swiss-Prot release 30, PIR release 41.10, Genpept release 85 and Brookhaven Protein Data Base release April 1994), respectively, was performed via an electronic mail implementation of FASTA (18) at EMBL Mail Services and BLAST at the National Center of Biotechnology Information (Bethesda, MD) (19). Secondary structure, solvent accessibility, and membrane-spanning regions were determined using Predict Protein at EMBL via electronic mail service. Membrane-spanning regions were predicted according to algorithms by Rao and Argos (21) and Klein (22). Sequence computation was done with the GCG package (GCG Program Manual for the Wisconsin Package, version 8, September 1994, Genetics Computer Group, Madison, WI).

Cloning of the 5' End of the hnl Gene—Total phage DNA of the H. brasiliensis cDNA library was prepared as described by Grossberger (23). The 5' end of hnl was cloned by two-step PCR. The following primers were used: primer 1, 5'-CCTCCAAGAACGTCAACAAG-3'; primer 2, 5'-CATCAAATGAGCCAATCTCC-3' with antisense orientation; vector-specific primer T3, 5'-AATTAACCCTCACTAAAGGG-3'.

PCR was performed in a total volume of 20 μl (25 pmol of primer, 0.2 mM dNTP, 1/3 reaction buffer (10 mM Tris, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, pH 8.8) and 2 units of Taq polymerase (Stratagene Cloning Systems, La Jolla, CA)) for 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C. The first linear amplification reaction contained primer 1 and 40 ng of DNA. In the second reaction primers 2 and T3 and 1/10 volume of reaction 1 as DNA template were used. The PCR products were purified and subcloned into plasmid pHNL-100, to yield pHNL-101.

A hnl fragment flanked by suitable restriction sites was constructed by replacing the 5' and 3' ends of the cloned hnl cDNA with PCR-generated fragments. Plasmid pHNL-101 containing the full-length hnl cDNA was used as the template.

The following primers were used: primer 1, 5'-CCTCCAAGAACGTCAACAAG-3'; primer 2, 5'-CATCAAATGAGCCAATCTCC-3' with antisense orientation; vector-specific primer T3, 5'-AATTAACCCTCACTAAAGGG-3'. PCR was performed in a total volume of 20 μl (25 pmol of primer, 0.2 mM dNTP, 1/3 reaction buffer (10 mM Tris, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, pH 8.8) and 2 units of Taq polymerase (Stratagene Cloning Systems, La Jolla, CA)) for 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C. The first linear amplification reaction contained primer 1 and 40 ng of DNA. In the second reaction primers 2 and T3 and 1/10 volume of reaction 1 as DNA template were used. The PCR products were purified and subcloned into plasmid pHNL-100, to yield pHNL-101.

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The type and Mutant Hnl cDNA—form plasmids pHNL-200 and pHNL-201. were subcloned into the respective sites of expression vector pSE420 to the resulting expression plasmids, pHNL-300, and the Cys81 site-direct mutation—Cys31 (TGT) was changed to Ser (TCT) with PCR, using plasmid pHNL-104 as DNA template and the following primers: gene-specific primer mutant primer in antisense orientation, 5'-CACATTGTATATGGTCTCAAGGTGG-3', containing a MvuI restriction site, and vector-specific primer, T3, 5'-AATTAACCTGACATTGA-3'. The PCR conditions were 25 pmol of primer, 0.2 mM dNTP, 1 x reaction buffer (10 mM Tris, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, pH 8.8) and 2 units of Taq polymerase (Stratagene, La Jolla, CA) for 30 cycles consisting of 1 min at 94 °C, 1 min at 52 °C, and 1 min at 72 °C in a reaction volume of 20 μl. The synthesized mutant DNA was cut with appropriate restriction enzymes, purified from an agarose gel and subcloned to obtain pHNL-105. The correct base replacement was confirmed by sequencing.

Construction of E. coli Expression Plasmid pHNL-200 and pHNL-201—The Ncol-HindIII fragments containing the entire coding region of the hnl wild-type gene (pHNL-103) and hnl mutant gene (pHNL-105) were subcloned into the respective sites of expression vector pSE420 to form plasmids pHNL-200 and pHNL-201.

Construction of S. cerevisiae Expression Plasmid Containing Wild-type and Mutant Hnl cDNA—The hnl wild-type cDNA fragment (pHNL-101) and the hnl mutant cDNA fragment (pHNL-105) were subcloned as BamHI fragments into the unique BglII cloning site of pHNL-103, resulting in plasmid pHNL-103. The correct nucleotide sequence was confirmed by sequence analysis.

Plasmid pHNL-103 was modified at the 3' end of the cDNA insert to introduce EcoRI and BamHI restriction sites. The HindIII-linearized pHNL-103 was ligated with a double-stranded adaptor (AGCTGAAATTGATCC; AGCTGGATCCGAAATTCA) obtaining construct pHNL-104. The presence of the adaptor sequence was confirmed by sequencing.

Protein expression and purification—Yeast strains containing the Hnl expression plasmid pHNL-300 (pHNL-104) and the hnl wild-type gene (pHNL-103) were subjected to a two-step ion exchange fast protein liquid chromatography on a Resource Q column (Pharmacia Biotech). The column was equilibrated with loading buffer (10 mM histidine/H₂SO₄, pH 6.7), and Hnl was eluted with a linear gradient of loading buffer/loading buffer plus 0.3 M (NH₄)₂SO₄. The protein was collected at a concentration of approximately 0.12 M protein/g of poly(A)⁺ RNA isolated from young leaves of H. brasiliensis was hybridized with digoxigenin-labeled hnl cDNA. hnl-specific mRNA of about 1130 nucleotides is marked by an arrow.

RESULTS

Cloning of Full-length hnl cDNA from H. brasiliensis—Young leaves of a 10-year-old H. brasiliensis tree containing hydroxynitrile lyase specific activity at about 0.4 units/mg of protein extract were the source of RNA used to construct the cDNA library. From 5 μg of poly(A)⁺ RNA a cDNA library with 2 x 10⁶ plaque-forming units per packaging reaction was obtained and amplified. The library contained more than 99% recombinants with an average insert size of 1000 base pairs. About 100,000 plaques were screened with rabbit anti-Hnl antiserum as described under "Materials and Methods." 12 positive phage clones were picked and converted into plasmids. The hybrid proteins expressed in E. coli were subjected to Western blot analysis, and extracts were assayed for Hnl activity. The clone harboring plasmid pHNL-100 expressed Hnl specific activity of 0.035 units/mg cytosolic protein and contained a 33-kDa fusion protein strongly reacting with anti-Hnl antiserum. The cDNA was sequenced, and an open reading frame of 255 amino acid residues downstream of the lacZ part was identified. Partial sequence analysis suggested that other immunoreactive dones may represent H. brasiliensis homo-
TABLE III

Heterologous expression of hnl cDNA in E. coli

Soluble cell extracts of E. coli strains expressing Hnl were prepared from noninduced and isopropyl-1-thio-β-D-galactopyranoside-induced E. coli strains XL1-Blue and Top10. Induction was carried out at the indicated temperatures. Cytosolic Hnl specific activity (IU/mg of protein) was determined in triplicate, with a standard deviation of less than ±10%.

| Construct                     | Host        | Cytosolic Hnl specific activity (IU/mg of protein) |
|-------------------------------|-------------|--------------------------------------------------|
|                               |             | Noninduced (37 °C) | Induced 22 °C | Induced 28 °C | Induced 37 °C |
| pSE420 (no insert)            | XL1-Blue    | <0.01               | ND          | <0.01       | <0.01       |
| pHNL-200 (Hnl-cDNA)           | XL1-Blue    | 0.03                | 0.61        | 0.14        | 0.06        |
| pHNL-200 (Hnl-cDNA)           | Top10       | 0.04                | ND          | 0.20        | 0.09        |
| pHNL-201 (Hnl-Ser81)          | XL1-Blue    | <0.01               | <0.01       | <0.01       | <0.01       |

* ND, not determined.

The full-length hnl cDNA was cloned by PCR as described under "Materials and Methods" and sequenced. Among the four characterized cDNA clones, which varied by 4 nucleotides at their 5’ ends, the longest cDNA clone extended the first isolate by 47 base pairs at the 5’-end. Two in-frame stop codons upstream of the open reading frame indicate that the complete hnl cDNA was isolated. Nucleotide sequence and the predicted amino acid sequence are shown in Fig. 1. The size of the cDNA of 1150 base pairs corresponds to the size of the hnl transcript of 1130 ± 30 nucleotides, as determined by Northern blot analysis (Fig. 2). The low frequency of immunoreactive clones in the expression library as well as the weak signal detected with 10 μg of poly(A) RNA suggest that hnl is only weakly expressed in leaves of H. brasiliensis that were used for our studies. Genomic DNA cleaved with different restriction endonucleases and probed with the isolated cDNA indicated that hnl is a unique gene with at least one intron that includes an EcoRI site (Fig. 3).

Characterization of the hnl cDNA—The open reading frame contained in the hnl cDNA encodes a protein with 257 amino acids with a predicted molecular mass of 29,227 Da. This prediction is in agreement with the plant protein subunit molecular weight of 30 kDa as determined by SDS-polyacrylamide gel electrophoresis.2 Proof that the cloned hnl gene encodes the Hnl enzyme was obtained by protein sequence analysis of prokaryotic fragments of Hnl purified from leaves of H. brasiliensis. Experimentally determined protein sequences are underlined in Fig. 1. The DNA sequence around the putative start ATG fulfills the known criteria for translation initiation sites of plant genes (27). The N terminus of the purified protein was blocked and therefore was not accessible to sequencing. No consensus sequences for N-glycosylation sites are present, supporting earlier findings that Hnl is unglycosylated.2

Functional expression of H. brasiliensis Hydroxynitrile Lyase in E. coli and S. cerevisiae—hnl was expressed in E. coli strains XL1-Blue and TOP10 under the control of the trc promoter. The Hnl enzyme activities in various preparations are summarized in Table III. Cytosolic Hnl depending on the E. coli strain and the temperature during induction with isopropyl-1-thio-β-D-galactopyranoside. An inverse correlation between induction rate and solubility of the heterologous protein was observed. Insoluble fractions were devoid of active protein but contained inactive Hnl that appeared to be cross-linked by intra- and intermolecular disulfide bridges. A broad smear of protein in the high molecular weight range was observed on nonreducing SDS-polyacrylamide gels in addition to the 30-kDa Hnl subunit (data not shown). Thus, high level protein expression may lead to the formation of inclusion bodies. After solubilization and refolding of protein from the insoluble fraction less than 5% of Hnl was recovered in an active form (Fig. 4). However, the presence of active enzyme in the heterologous system provides further definitive proof that the cloned gene encodes Hnl.

Hnl was also expressed in S. cerevisiae. In this host system, Hnl could be obtained in high yield in a soluble form of up to 20% of the cytosolic protein. Recombinant Hnl purified from S. cerevisiae had a specific activity of 22 ± 3 IU/mg, which is similar to the specific activity of 18 ± 3 for the enzyme that was isolated from H. brasiliensis leaves (Table IV).

Protein Sequence Analysis—(S)-Hydroxynitrile lyase from H. brasiliensis shares 74% identity with Hnl from another Euphorbiaceae species, Manihot esculenta (28). Both enzymes are members of the class of non-FAD hydroxynitrile lyases. 35% identity with two proteins of unknown function from Oryza sativa (29) suggests functional or structural relationships of these proteins to Hnl. No significant homology to (R)-hydroxynitrile lyase (FAD) from Prunus serotina (7) or (S)-Hnl (non-FAD) from S. bicolor (10) is evident.

Hydroxynitrile lyases from H. brasiliensis and M. esculenta, and also the two Oryza proteins of unidentified function, share weak homology with the C termini of soluble mammalian epoxide hydrolases (30). These enzymes have been classified as new members of the α/β-hydrolase fold protein family, containing Asp-Asp-His as the catalytic triad residues (31). Despite low primary sequence similarity members of this protein fam-
ily show a similar three-dimensional fold and conserved sequence order for the catalytic triad residues (32). Based on sequence homology to soluble mammalian epoxide hydrolases with its postulated catalytic triad, a potential catalytic triad Ser-Asp-His is suggested for the H. brasiliensis and M. esculenta enzymes (Fig. 5). (S)-Hydroxynitrile lyase from S. bicolore (10) has significant homology to various serine-carboxypeptidases, further supporting the notion of an active site serine residue. However, the same region around Ser80 in the H. brasiliensis enzyme also contains the aldehyde dehydrogenase motif, indicating a potential catalytic role of Cys81. Hnl of several species of Prunus (8, 33, 34) and of Ximenia americana (35) and Sorghum vulgare (36) are inhibited by thiol reagents. Also, a cysteine residue can be chemically modified with pseudosubstrates such as α,β-unsaturated propiophenones. Furthermore, the putative active center of Hnl from H. brasiliensis shares significant homology to the lipase and carboxypeptidase active site patterns (Fig. 6). These patterns contain serine or cysteine as the catalytically active residues, which are both present in Hnl. Determination of the Active Site of Hnl—In order to further investigate the potential catalytic role of Cys81, we replaced it with serine. The mutated protein, Hnl-Ser81, was expressed in E. coli and S. cerevisiae. Hnl activity in cytosolic protein extracts was completely abolished in E. coli (Table III), suggesting that Cys81 is essential for catalytic activity. Since Hnl expression in E. coli resulted in rather low levels of active enzyme, we also expressed wild-type and mutated Hnl in S. cerevisiae. High expression levels (20% of cytosolic fraction) were achieved in this system. Mutated Hnl, Hnl-Ser81, was expressed at the same level as the wild-type enzyme. No differences in the electrophoretic mobility between the mutant and wild-type protein were observed on native polyacrylamide and isoelectric focusing gels (data not shown), indicating that the amino acid substitution did not cause major structural alterations. As can be seen from the data summarized in Table IV, the specific activity of the purified Hnl-Ser81 protein was less than 2% of the specific activity determined for the wild-type Hnl protein. This further supports our view of a possible involvement of Cys81 in Hnl catalysis.

Inhibition studies (Table II) support a potential role of SH groups in catalysis. Hg(II) chloride and parachloromercuribenzoate completely inactivated the enzyme, whereas more bulky sulfhydryl reagents like N-ethylmaleimide or E-64 (N-[1-3-trans-carboxyoxiran-2-carbonyl]-1-leucyl)-agmatin) had no effect on Hnl activity. These data suggest that the active center...
molecular biology as well as in biocatalytic applications. Here we report for the first time the cloning and molecular characterization of a class II enzyme from H. brasiliensis that accepts both aliphatic and aromatic (S)-cyanhydrins as substrates (11). Hnl from H. brasiliensis consists of a single polypeptide of 257 amino acids that does not appear to require extensive post-translational modification such as N-glycosylation for activity. This is in marked contrast to (R)-hydroxynitrile lyases, which are highly glycosylated, and to (S)-hydroxynitrile lyase from S. bicolor, a glycoprotein that requires post-translational proteolytic processing for activity (10). The N terminus of the H. brasiliensis protein appears to be blocked; by analogy to the M. esculenta enzyme and by applying rules for the N-terminal structure of intracellular proteins in eukaryotes (38) the N-terminal amino acid should be an acetylated alanine. The N-terminal 20 amino acid residues are hydrophobic and are predicted to adopt a β-fold conformation. We suggest that this region of the protein may act as a dimerization signal rather than as a membrane anchor, since we found that the soluble protein exists as a homodimer in low salt buffer.2

Protein sequence analysis revealed high overall homology of the H. brasiliensis enzyme to Hnl from M. esculenta and moderate but significant homology to two proteins from rice (O. sativa). One of the rice proteins is induced after infiltration of leaves with Pseudomonas syringae, leading to speculation that it may be involved in a resistance response. Although sequence comparison suggested a lipase function (29), based on the structural considerations derived from the present study it is tempting to speculate that the rice protein may function as a hydroxynitrile lyase.4

Although class I and class II hydroxynitrile lyases catalyze similar reactions, the overall molecular organization and functionalization of the enzymes and their active sites appear to be completely different. Furthermore, within the class II enzymes two subgroups can be proposed, one comprising the glycosylated, carboxypeptidase-like Sorghum type and the other a nonglycosylated, unprocessed Euphorbiaceae type. Enzymes of the two subgroups indeed differ in their active site architecture. Active site residue Cys81, which is essential for the H. brasiliensis enzyme, is absent in the proposed active site region of the S. bicolor enzyme (10). Inhibition of Sorghum sp. Hnl by mercury chloride (36) may be due to a disintegration of disulfide bridges required to establish heterotetrameric structure rather than inactivation of an active site cysteine residue. Lack of sequence homology among Hnls from different plant families indicates that these enzymes may have evolved convergently from different precursor structures. Thus, nature has adjusted enzyme activities for the retroaddition of HCN to a carbonyl group most likely resulting in slightly different mechanistic solutions. This may also explain the heterogeneous substrate specificities and stereoactivities of the various Hnls.

Hydroxynitrile lyase from H. brasiliensis was expressed in E. coli despite its rather low codon bias index for this host. Under the conditions of our expression system Hnl protein aggregated and formed inclusion bodies that could not be efficiently refolded into an active form by standard methods. In contrast, substantial overexpression of active Hnl could be achieved in the yeast S. cerevisiae, which thus appears to be a much more efficient host system for heterologous Hnl production.

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REFERENCES

1. Conn, E. E. (1984) in Annual Proceedings of the Phytochemical Society of Europe (Boudet, A. M., Alibert, G., Marigo, G., and Lea, P. J., eds), Vol. 24, pp. 1–28. Oxford University Press.
2. Rosenthaler, L. (1988) Biochim. Biophys. Acta 13, 238–253.
3. Nahrstedt, A. (1981) Plant Syst. Evol. 150, 35–47.
4. Lieberei, R. (1988) Phytopathol. UAR 122, 134–146.
5. Saitoh, D. (1996) Cyanogenesis in Higher V. W. Weige zur Metabolisierung Cyanogenester. Ph.D. thesis, University of Braunschweig, Germany.
6. Kruse, C. G. (1992) in Chirality in Industry (Collins, A. N., Sheldrake, G. N., and Croddy, J., eds) pp. 279–299. John Wiley & Sons, Inc., New York.
7. Croddy, J. P., and Bolton, P. J. (1993) Plant Cell Physiol. 34, 1339–1443.
8. Jannick, L., and Preun, J. (1984) Eur. J. Biochem. 138, 319–325.
9. Petrosian, I. P., Goldberg, J., and Bush, E. J. (1994) Biochemistry 33, 2893–2899.
10. Wajant, H., Mundry, K. W., and Pfizenmaier, K. (1994) Plant Mol. Biol. 26, 735–746.
11. Kleinjung, N., Griengl, H., and Hayn, M. (1993) Tetrahedron Lett. 34, 4769–4772.
12. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) Eur. J. Biochem. 153, 163–167.
13. Kingsman, A. J. (1990) Methods Enzymol. 185, 329–341.
14. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1990) Current Protocols in Molecular Biology, Vols. 1 and 2, Greene Publishing Associates and Wiley-Interscience, New York.
15. Chirgwin, J. M., Przybyla, A. E., McDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299.
16. Avis, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1408–1412.
17. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.
18. Pearson, W. R., and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2444–2448.
19. Altschul, S. F., Gish, W., Miller, W., and Meyers, E. W. (1990) J. Mol. Biol. 215, 403–410.
20. Rost, B., and Sander, C. (1993) J. Mol. Biol. 232, 584–599.
21. Rao, J. K. M., and Argos, P. (1986) Biochim. Biophys. Acta 869, 197–214.
22. Klein, P., Kanihase, M., and Delisi, C. (1985) Biochim. Biophys. Acta 815, 468–476.
23. Groesserberger, D. (1987) Nucleic Acids Res. 15, 6737.
24. Martson, F. A. O. (1987) in DNA Cloning: A Practical Approach (Glover, D. M., ed) Vol. III, pp. 59–88. IRL Press, Oxford.
25. King, J., and Lanenm, K. U. (1971) J. Mol. Biol. 62, 465–477.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.

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R. Dudler, personal communication.
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27. Lütcke, H. A., Chow, K. C., Middel, F. S., Moss, K. A., Kern, H. F., and Scheele, G. A. (1987) EMBO J. 6, 43–48
28. Hughes, J., Carvalho, F. J. P. de C., and Hughes, M. A. (1994) Arch. Biochem. Biophys. 311, 496–502
29. Relman, C., Hoffmann, C., Mauch, F., and Dudler, R. (1995) Physiol. Mol. Plant Pathol. 46, 71–81
30. Beetham, J. K., Tian, T., and Hammock, B. D. (1993) Arch. Biochem. Biophys. 305, 197–201
31. Arndt, M., Grant, D. F., Beetham, J. K., Oesch, F., and Hammock, B. D. (1994) FEBS Lett. 338, 251–256
32. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, J. S., Silman, I., Schrag, J., Sussman, J. L., Verschueren, K. H. G., and Goldman, A. (1992) Protein Eng. 5, 197–211
33. Yemm, R. S., and Poulton, J. E. (1968) Arch. Biochem. Biophys. 250, 322–328
34. Xu, L. L., Singh, B. K., and Conn, E. E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6978–6981
35. Kuroki, G. W., and Conn, E. E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6978–6981
36. Bove, C., and Conn, E. E. (1961) J. Biol. Chem. 236, 207–236
37. Hecht, H. J., Schek, H., Haag, T., Pfeifer, O., and van Pee, K. H. (1994) Struct. Biol. 1, 532–537
38. Kendall, R. L., Yamada, R., and Bradshaw, R. A. (1990) Methods Enzymol. 185, 398–407
39. Gerdts, E., and Pfeifer E. (1972) Hoppe-Seyler’s Z. Physiol. Chem. 353, 271–286
40. Xu, L. L., Singh, B. K., and Conn, E. E. (1988) Arch. Biochem. Biophys. 263, 256–263
41. Albrecht, J., Jansen, I., and Kula, M. R. (1993) Biotechnol. Appl. Biochem. 17, 191–203