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Supporting Information

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A New (*R*)-Selective Hydroxynitrile Lyase from *Arabidopsis thaliana* with an α/β-Hydrolase Fold

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1. Cloning and expression of *At*HNL

The *At*HNL-gene was amplified from *Arabidopsis* cDNA by polymerase chain reaction (PCR). Specific primers were derived from the sequence in the database (gene bank entry AAN13041) including appropriate restriction sites for cloning of the gene into the vector pET28a (Novagen).

5'-primer: TATA*CCATGG*AGAGGAAACATCACTTCGTGTTAGTTCACA
NcoI site

3'-primer: TATA*CTCGAG*TTACATATAATCGGTGGCAATAGCAGAGAG
XhoI site

The PCR reaction was carried out in a thermo cycler (Eppendorf) with an annealing temperature of 58°C.

E. coli BL21(DE3) was chosen as a host for enzyme expression. After transformation of the final plasmid p*hnlA* by electroporation the cells were grown in shaking flasks at 37°C until an optical density at 580 nm of 0.7 was reached, and protein expression was started by addition of 0.4 mM isopropyl-β-D-thiogalactopyranosid (IPTG). Subsequently, cells were grown for further 16 h at 30°C. Protein expression was followed by SDS-PAGE analysis (Fig. 1). Cells were harvested and lysated by ultrasonification. Analysis of soluble and insoluble fractions revealed that 70-80% of the protein is in the soluble fraction (lane 3, Fig. 1).

For production of large amounts of *At*HNL a standard fed-batch cultivation was performed.[1] From a 15 L cultivation 1.75 kg cells were obtained with an activity of ca. 2300 U/g cells. Thus, in 1.75 kg cells a total of 4,046,000 Units *At*HNL was produced. Activity measurements were performed with mandelonitrile according to Bauer et al.[2]

1 Unit is defined as the amount of enzyme which cleaves 1 µmol mandelonitrile in 1 min at pH 5 and 25°C.

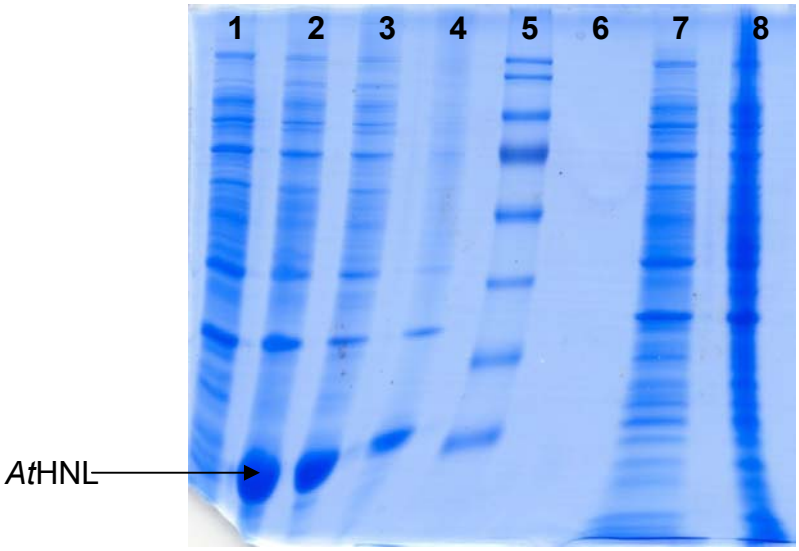

Figure 1: Expression of *At*HNL in BL21(DE3), as negative control BL21(DE3) with pET28a was used. **Lane 1**: whole cells 0 h after induction, **lane 2**: whole cells 16 h after induction, **lane 3**: soluble fraction, **lane 4**: insoluble fraction, **lane 5**: protein standard (prestained protein ladder, Fermentas), **lane 6**: empty, **lane 7**: negative control 0 h after induction, **lane 8**: negative control 16 h after induction.

2. Purification of *At*HNL

Cells with AtHNL were broken by ultrasonification, and the resulting crude cell extract was first desalted by a size-exclusion chromatography on Sephadex G25. The desalted crude extract was subsequently purified by anion exchange chromatography (Q-Sepharose fast flow) using 50 mM potassium phosphate buffer, pH 7.5. *At*HNL was eluted with a NaCl gradient at about 100 mM NaCl. *At*HNL-containing fractions are pooled, desalted and concentrated, yielding a purity of about 80%. For further purification size exclusion chromatography (Sephadex G-200 column) was applied.

3. Creation of *At*HNL variants by site-directed mutagenesis

Point mutations were introduced using the QuickChange PCR protocol from Stratagene (Quikchange® II Site Directed Mutagenesis Kit). For amplification *Pfu*-Turbo polymerase from Stratagene was employed; p*At*HNL, containing the *At*HNL-gene in the vector pET28a (Novagen) was used as a template and mutagenesis primers are summarized in Table 1.

Table 1: Primers to introduce point mutations into p*At*HNL:

Variant	**5' Primer**	**3' Primer**
Ser81Ala	GGTAATTCTGGTTGGATTCGCGTTCGGAGGC ATCAAC	GTTGATGCCTCCGAACGCGAATCCAACCAG AATTACC
Asp208Asn	CGTAATGAGTAGTGAAAACAAAGCCATCCCCT GC	GCAGGGGATGGCTTTGTTTTCACTACTCATT ACG
His236Phe	CGATGGCGGAGATTTTATGGTGATGCTCTCC	GGAGAGCATCACCATAAAATCTCCGCCATC G
Tyr14Cys	CGTGTTAGTTCACAACGCTTGCCATGGAGCC TGG	CCAGGCTCCATGGCAAGCGTTGTGAACTAA CACG

4. Formation of cyanohydrins

4.1 HCN formation: The required amount of HCN was freshly distilled in a well ventilated hood.

General procedure: 4 g of sodium cyanide were dissolved in 10 ml deionized water and 10 ml of 5 mol/l sulphuric acid were added drop wise within 2 minutes. Afterwards the reaction mixture was heated up to 75°C and formed HCN was trapped and stored at 5°C. For the removal of water traces a spatula tip of sodium sulphate was added. All waste solutions were collected and disposed. An electrochemical HCN-detector (Micro III G203, GfG-Gesellschaft für Gerätebau mbH, Dortmund, Germany) was placed into the hood for continuous monitoring.

4.2 General experimental setup for cyanohydrin synthesis: Syntheses of cyanohydrins were performed either with crude cell extracts or purified *At*HNL. A biphasic system consisting of diisopropylether and 50 mmol/l citrate-phosphate buffer pH=5.0 (ratio 1:1) was applied with 50 mmol/l carbonyl compound, 250 mmol/l HCN, and 40 U/ml *At*HNL (based on the assay described in section 1). The reactions were carried out at 10°C in 8 ml glass vials, which were shaken horizontally. The reaction was followed by taking samples (50 µl) from the organic phase which were added to a mixture of 700 µl dichloromethane, 50 µl trifluoroacetic anhydride, and 50 µl pyridine for the acetylation procedure (GC). For the determination of *n*-hexanal- and *n*-decanal-cyanohydrin heptafluorobutyric acid anhydride was used instead of trifluoracetic anhydride. Sampling was performed after 3 h, 6 h, and 22 h. All experiments were repeated three times and the mean and standard deviation were calculated. The latter was between 5 to 10%.

The conversion of carbonyl compounds to the corresponding cyanohydrins (as trifluoroacetyl-derivatives) as well as the enantiomeric excesses were determined by gas chromatographic analysis using a Chiraldex capillary gas chromatography column (G-PN-gamma-Cyclodextrin, propionyl) (Astec, USA) on a CP3800 gas chromatograph (Varian, USA) with a flame ionization detector (FID) with helium as a carrier gas (2 ml/min). The column temperature was kept constant at 110°C, except for *m*-phenoxybenzaldehyde-cyanohydrin which was determined at a column temperature of 130°C, and a carrier gas (helium) flow of 4 ml/min. For *o*-iodine-benzaldehyde cyanohydrin a carrier gas flow of 8 ml/min, and for *m*-iodine-benzaldehyde cyanohydrin a carrier gas flow of 6 ml/min at a column temperature of 110 °C was applied. Temperatures of the injector and detector were set to 250°C in all cases.

5. Sequence alignment of *At*HNL, *Me*HNL and *Hb*HNL

The sequence alignment (Figure 2) was performed using the program ClustalW (http://www.ebi.ac.uk/clustalw/). *At*HNL shows 70% sequence similarity and 45-47% sequence identity relative to *Hb*HNL and *Me*HNL.

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MeHNL           -MVTAHFVLIHTICHGAWIWHKLKPALERAGHKVTALDMAASGIDPRQIEQINSFDEYSE 59
HbHNL           -MAFAHFVLIHTICHGAWIWHKLKPLLEALGHKVTALDLAASGVDPRQIEEIGSFDEYSE 59
AtHNL           MERKHHFVLVHNAYHGAWIWYKLKPLLESAGHRVTAVELAASGIDPRPIQAVETVDEYSK 60
                     ****:*.  ******:**** **  **:***:::****:*** *: : :.****:

MeHNL           PLLTFLEKLPQGEKVIIVGESCAGLNIAIAADRYVDKIAAGVFHNSLLPDTVHSPSYTVE 119
HbHNL           PLLTFLEALPPGEKVILVGESCGGLNIAIAADKYCEKIAAAVFHNSVLPDTEHCPSYVVD 119
AtHNL           PLIETLKSLPENEEVILVGFSFGGINIALAADIFPAKIKVLVFLNAFLPDTTHVPSHVLD 120
                **:  *: ** .*:**:** * .*:***:*** :  ** . ** *:.**** * **:.::

MeHNL           KLLESFPDWRDTEYFTFTNITGETITTMKLGFVLLRENLFTKCTDGEYELAKMVMRKGSL 179
HbHNL           KLMEVFPDWKDTTYFTYT-KDGKEITGLKLGFTLLRENLYTLCGPEEYELAKMLTRKGSL 178
AtHNL           KYMEMPGGLGDCEFSSHETRNG-TMSLLKMGPKFMKARLYQNCPIEDYELAKMLHRQGSF 179
                * :*   .  *  : :.    *  :: :*:*  ::: .*:  *   :******: *:**:

MeHNL           FQNVLAQRPKFTEKGYGSIKKVYIWTDQDKIFLPDFQRWQIANYKPDKVYQVQGGDHKLQ 239
HbHNL           FQNILAKRPFFTKEGYGSIKKIYVWTDQDEIFLPEFQLWQIENYKPDKVYKVEGGDHKLQ 238
AtHNL           FTEDLSKKEKFSEEGYGSVQRVYVMSSHDKAIPCDFIRWMIDNFNVSKVYEIDGGDHMVM 239
                * : *:::  *:::****::::*: :.:*: :  :*  * * *:: .***:::**** :

MeHNL           LTKTEEVAHILQEVADAYA 258
HbHNL           LTKTKEIAEILQEVADTYN 257
AtHNL           LSKPQKLFDSLSAIATDYM 258
                *:*.::: . *. :*  *
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Figure 2: Sequence alignment of *At*HNL, *Me*HNL and *Hb*HNL. Residues of the catalytic triad are marked by a frame. Residues of the substrate binding site, which are assumed to be responsible for the stereoselectivity, are underlined.

6. Creation of the structural model of *At*HNL

Based on the recently published structure of the (*S*)-selective hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL, pdb-code 1YB6) a homology model of the (*R*)-selective hydroxynitrile lyase from *Arabidopsis thaliana* (*At*HNL) was created using the Swiss-model server.[3] The catalytic triad is correctly aligned within the α/β-hydrolase fold, and the overall RMSD of the backbone Cα-atoms is only 0.2 Å which supports the reliability of the predicted 3D-model.

Comparison of active site structures and analysis of the differences by modelling (*R*)- and (*S*)-mandelonitrile into the *At*HNL homology model were performed using the program

MOLOC.[4] The structures in complex with the substrate were subsequently minimized and relaxed by molecular dynamics to analyse the geometry of the active site with bound substrate, since we started from a homology model without bound ligand.

References:

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[3] T. Schwede, J. Kopp, N. Guex, M. C. Peitsch, *Nucleic Acids Res.* **2003,** *31*, 3381.

[4] a) P. R. Gerber, *J. Comput.-Aided Mol. Des*. **1998**, *12*, 37; b) P. R. Gerber, K. Müller, *J. Comput.-Aided Mol. Des*. **1995**, *9*, 251.