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Uneven twins: Comparison of two enantiocomplementary hydroxynitrile lyases with α/β-hydrolase fold

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ABSTRACT

Hydroxynitrile lyases (HNLs) are applied in technical processes for the synthesis of chiral cyanohydrins. Here we describe the thorough characterization of the recently discovered *R*-hydroxynitrile lyase from *Arabidopsis thaliana* and its *S*-selective counterpart from *Manihot esculenta* (*Me*HNL) concerning their properties relevant for technical applications. The results are compared to available data of the structurally related *S*-HNL from *Hevea brasiliensis* (*Hb*HNL), which is frequently applied in technical processes. Whereas substrate ranges are highly similar for all three enzymes, the stability of *Me*HNL with respect to higher temperature and low pH-values is superior to the other HNLs with α/β-hydrolase fold. This enhanced stability is supposed to be due to the ability of *Me*HNL to form tetramers in solution, while *Hb*HNL and *At*HNL are dimers. The different inactivation pathways, deduced by means of circular dichroism, tryptophan fluorescence and static light scattering further support these results. Our data suggest different possibilities to stabilize *Me*HNL and *At*HNL for technical applications: whereas the application of crude cell extracts is appropriate for *Me*HNL, *At*HNL is stabilized by addition of polyols. In addition, the molecular reason for the inhibition of *Me*HNL and *Hb*HNL by acetate could be elucidated, whereas no such inhibition was observed with *At*HNL.

1. Introduction

Hydroxynitrile lyases (HNL, EC 4.1.2.X) catalyze the cleavage of cyanohydrins into a carbonyl compound and HCN, which represents the second step in cyanogenesis and acts as a plant defence mechanism against microbial and herbivore attack. The reverse reaction is used in biotechnological processes for the production of chiral cyanohydrins, which are versatile chiral building blocks in pharmaceutical and agrochemical industry (Fechter and Griengl, 2004; Purkarthofer et al., 2007; Sharma et al., 2005).

The availability of several *R*- and *S*-selective enzymes allows the production of a broad range of chiral cyanohydrins. The most frequently used enzymes in technical processes are the *R*-HNL from *Prunus amygdalus* (bitter almond) (Glieder et al., 2003) and the *S*-HNLs from *Hevea brasiliensis* (para rubber tree) (Hasslacher et al., 1996b; Purkarthofer et al., 2007). Besides, the *S*-HNL from *Manihot esculenta* (cassava) (Hughes et al., 1994) is also used in few technical applications (Daussmann et al., 2006).

HNLs are a well known example for convergent enzyme evolution, generating a common enzymatic activity in different structural frame works. Until recently it was assumed that *R*-selective enzymes are derived from oxidoreductase precursors, whereas *S*-selective enzymes belong to the structural class of α/β-hydrolases (Ollis et al., 1992). The first exception from this rule was recently discovered with a new HNL (*At*HNL) in *Arabidopsis thaliana* (mouse-ear cress) (Andexer et al., 2007), which contains several genes with high sequence similarity to HNLs with an α/β-hydrolase fold derived from *H. brasiliensis* (*Hb*HNL) and *M. esculenta* (*Me*HNL) (Wäspi et al., 1998). One of these gene products (*At*HNL) shows pronounced HNL-activity with respect to the cleavage and formation of chiral cyanohydrins (Andexer et al., 2007). Despite the striking sequence similarity of 45% identical and 68% similar amino acid residues relative to the *S*-selective *Hb*HNL and *Me*HNL, *At*HNL is strictly *R*-

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selective. The putative active site residues serine-81, aspartate-208 and histidine-236 forming the catalytic triad were confirmed by site-directed mutagenesis (Andexer et al., 2007) and preliminary results regarding the crystal structure (unpublished results) additionally prove that *At*HNL belongs to the family of α/β-hydrolases.

Beside the substrate range of the synthesis reaction, which was extensively described for *Pa*HNL (Dreveny et al., 2002; Weis et al., 2004) as well as for all three α/β-hydrolase enzymes (Schmidt et al., 1996; Andexer et al., 2007; Förster et al., 1996), a detailed knowledge about the catalysts' stability and inactivating parameters is important. Such data are a prerequisite to design optimal reaction conditions for technical applications or to overcome limitations by mutagenesis, as was demonstrated for the most widely used HNLs from *P. amygdalus* and *H. brasiliensis* (Hickel et al., 1997; Glieder et al., 2003). In contrast to this, only few and incomplete data are available for the HNL from *M. esculenta*, whereas such data are complete missing for *At*HNL. To fill this gap, these enzymes were comparatively characterized concerning parameters relevant for application, such as substrate range, kinetic behaviour, influences of different buffer salts, pH and temperature on stability and activity. Emphasis was laid on the stability and inactivation mechanisms at low pH, as the enzymatic production of most cyanohydrins requires low pH-values and temperatures below 10 °C to keep the products stable (Cholod, 1993) and to suppress the unselective chemical side reaction (Bühler et al., 2003; Kragl et al., 1990; Niedermeyer and Kula, 1990; Willeman et al., 2000).

2. Materials and methods

2.1. Preparation of Arabidopsis cDNA

cDNA was prepared from mRNA from *Arabidopsis* seedlings (kindly provided by the institute for botany IV, University of Düsseldorf) with the "RevertAid™ First Strand cDNA Synthesis Kit" (Fermentas).

2.2. Cloning of AtHNL and MeHNL

The genes of interest were amplified from cDNA (*At*HNL) respective an existing construct (*Me*HNL) by PCR with specific primers (*At*HNL: 5′:TATACCATGGAGAGGAAACATCACTTCGTGTTAGTTCACA, 3′:TATACTCGAGTTAC ATATAATCGGTGGCAATAGCAGAGAG; *Me*HNL: 5′:ATATTCTAGAAATAATTTTG TTTAACTTTAAGAAGGAGATATACCATGGTAACTGCACATTTTTT, 3′:ATATCTCGA GTTATCAAGCATAAGCATCAGC) and cloned into pET28a (Novagen) vectors. PCRs were performed according to a standard protocol using either Turbo Pfu Polymerase (Stratagene) or Phusion Polymerase (Finnzymes). Amplified genes were restricted with respective restriction endonucleases (Fermentas) and ligated with the equally restricted vectors (T4 DNA ligase, Fermentas) according to the manufacturer's instructions. Genes were sequenced (Sequiserve, Vaterstetten) prior to transformation of competent *E. coli* BL21(DE3) cells via electroporation.

2.3. Expression

An over-night culture (LB-medium + kanamycin (50 μg/mL) (pET28a) or ampicilline (100 μg/mL) (pET22b)) was inoculated with a single colony and incubated for 16 h at 37 °C. The main culture was inoculated with the overnight culture (1:20) and induced with IPTG (0.4 mM) when an optical density at 580 nm of 0.6 was reached. After 20 h growth at 25 °C, 150 rpm, cells were harvested and stored at −20 °C. Expression was checked by SDS–polyacrylamide gel electrophoresis. For separation of soluble and insoluble proteins, cells were lysated by sonication and centrifuged for 20 min at 14,000 rpm. To obtain larger amounts of cells, BL21(DE3)_p*At*HNL and BL21(DE3)_p*Me*HNL were fermented using a standard fed-batch fermentation protocol (Korz et al., 1995). From a 15 L fermentation 1.95 kg (*Me*HNL) and 1.75 kg (*At*HNL) cell were harvested, respectively, containing a total activity of 2 GU (*Me*HNL) and 2 GU (*At*HNL), respectively (measured with crude cell extracts using the mandelonitrile cleavage assay).

2.4. Purification of recombinant proteins

2.4.1. AtHNL

20 g BL21(DE)_p*At*HNL cells were resuspended in potassium phosphate buffer (50 mM, pH 6) and lysated by sonication (4 × 5 min at 70 W/cm^2 on ice with an ultrasonic processor UP200S and a sonotrode S14D (Dr. Hielscher GmbH)). After centrifugation (35,000 × *g*, 4 °C, 45 min), the resulting crude extract (ca. 30 ml) was desalted on Sephadex G-25 (1 L bed volume, potassium phosphate buffer (10 mM, pH 6)). Subsequently, anion exchange chromatography on Q-Sepharose (column: 25 ml bed volume) was performed, which was equilibrated with potassium phosphate buffer (50 mM, pH 6; buffer A). After elution of non-bound proteins, *At*HNL containing fractions were eluted with a linear NaCl gradient in the same buffer (buffer B: buffer A + 1 M NaCl). *At*HNL-containing fractions eluted with a NaCl concentration of 150 mM. Combined fractions with HNL-activity were desalted on a Sephadex G-25 column (1 L bed volume, potassium phosphate buffer (10 mM, pH 6)) and subsequently lyophilized or concentrated by pressure dialysis with a Diaflo YM10 filter (Amicon) to a final protein concentration of 10 mg/ml. Protein determination was performed according to Bradford (1976). Purified *At*HNL (90% purity) exhibits a specific activity of 70–90 U/mg toward mandelonitrile.

2.4.2. MeHNL

10 g BL21(DE3)_p*Me*HNL cells were resuspended in potassium phosphate buffer (40 ml, 10 mM, pH 7.5), and treated as described for *At*HNL. For desalting of the crude cell extract potassium phosphate (10 mM, pH 7.5) was used. Fractions with HNL activity were loaded on a Q-Sepharose anion-exchange column (bed volume 27 ml) (Amersham Biosciences), which was equilibrated with potassium phosphate (10 mM, pH 7.5). *Me*HNL was eluted with a potassium phosphate gradient (10–50 mM, pH 7.5). One fraction of the active peak, eluted at 50 mM potassium phosphate, was lyophilized and stored at −20 °C. The residual part was concentrated by pressure dialysis with a Diaflo YM10 filter (Amicon) to a final protein concentration of 7 mg/ml. The purified protein (95% purity) has a specific activity of ca. 40–60 U/mg towards mandelonitrile. *Me*HNL-Cys81Ala was purified according to the procedure established for wildtype *Me*HNL. *E. coli* cells expressing *Me*HNL-Cys81Ala were kindly provided by Codexis. The purified protein (95% purity) showed a specific activity of 62 U/mg towards the cleavage of mandelonitrile.

2.5. Assays for hydroxynitrile lyase activity

2.5.1. Cleavage of mandelonitrile in aqueous medium

The increase of the benzaldehyde concentration was measured continuously at 280 nm in quartz glass cuvettes following a published protocol (Hanefeld et al., 2001). In brief: citrate phosphate buffer (700 μl) (100 ml contain: 24.3 ml of 0.1 M citric acid, 0.2 M K_2HPO_4, ad. 100 ml deionized water, final pH 5.0) is mixed with the enzyme solution (100 μl) in potassium phosphate buffer (10 mM, pH 6). The reaction was started by addition of the mandelonitrile solution (200 μl; 67 mM mandelonitrile in citrate phosphate buffer, pH 3.5) and monitored for 1 min. Subsequently, the activity was calculated using the molar extinction coefficient of benzaldehyde (1376 L mmol^{-1} cm^{-1}).

One unit of HNL activity is defined as the amount of enzyme which converts 1 μmol mandelonitrile per minute in citrate phosphate buffer, pH 5, 25 °C. All measurements were performed with a minimum of triplicates; blanks with all components except HNL were always determined twice. To determine kinetic parameters it was necessary to increase the amount of substrate in the assay to >15 mM. To achieve this, the assay composition was changed as follows: citrate phosphate buffer (100 μl, pH 5), enzyme solution (100 μl), mandelonitrile solution (different mandelonitrile concentrations, 800 μl); with this setup substrate concentrations up to 53 mM are possible. Data from kinetic measurements were fitted using the program ORIGIN 7G (OriginLab Corporation), for both cleavage reactions and *Me*HNL-catalyzed formation of mandelonitrile the standard Michaelis–Menten equation was used. In contrast, the *At*HNL-catalyzed synthesis of mandelonitrile was fitted with a formula including substrate surplus inhibition and cooperativity:

$$V = \frac{V_{max} \cdot [S]^h}{K_s^h + S^h + (S^2/K_I)^h}$$

V is the velocity (U), V_{max} is the maximal velocity (U), $[S]$ is the substrate concentration (mM), K_S is the equilibrium constant, h is the Hill coefficient, and K_I is the inhibition constant.

2.6. Cleavage of further cyanohydrins

The substrate range for the cleavage reaction was investigated using a microtiter plate assay based on the detection of HCN (Andexer et al., 2006). Commercial available cyanohydrins (acetone cyanohydrin, lactonitrile, cyclohexanone cyanohydrin, *m*-phenoxy-benzaldehyde cyanohydrin, and propionaldehyde cyanohydrin) were employed as substrates, which were in some cases (e.g. acetone cyanohydrin) of technical grade quality and contained varying amounts of the corresponding carbonyl compounds.

2.7. Synthesis reaction

Preparation of HCN: The required amount of HCN was freshly distilled in a well ventilated hood. Sodium cyanide (4 g) was dissolved in deionized water (10 ml) and sulphuric acid (5 M, 10 ml) was added drop wise within 2 min. 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