ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 6 (1999) 315–332

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Cloning and expression of (*R*)-hydroxynitrile lyase from *Linum usitatissimum* (flax) [1]

Holger Breithaupt [a], Martina Pohl [a], Wolfram Bönigk [b], Petra Heim [a], Karl-Ludwig Schimz [c], Maria-Regina Kula [a,*]

[a] *Institut für Enzymtechnologie, Universität Duesseldorf, Forschungszentrum Jülich, D-52426 Jülich, Germany*
[b] *Institut für Biologische Informationsverarbeitung 1, Forschungszentrum Jülich, D-52428 Jülich, Germany*
[c] *Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52428 Juelich, Germany*

Received 9 February 1998; revised 14 April 1998; accepted 14 April 1998

Abstract

The gene encoding for (*R*)-hydroxynitrile lyase ((*R*)-HNL) from *Linum usitatissimum* has been cloned by polymerase chain reaction using 3′,5′-RACE (rapid amplification of cDNA ends). The resulting clone contained an open reading frame of 1266 bp corresponding to a protein of 422 amino acids (45.8 kDa), which shows significant homologies to zinc-dependent formaldehyde dehydrogenases and alcohol dehydrogenases from various organisms. The dimeric active enzyme was expressed in *Escherichia coli* as N-terminal hexa-histidine fusion protein allowing the purification of homogeneous protein in one step. The formation of inclusion bodies could be reduced using a thioreductase deficient *E. coli* strain as a host and performing expression of (*R*)-HNL at 28°C. Under these conditions recombinant (*R*)-HNL was obtained with a specific activity of 76 U/mg. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Cloning; Dehydrogenase; Expression; Hydroxynitrile lyase; His-tag; Inclusion bodies; *Linum usitatissimum*; Oxynitrilase; Protein purification; RACE

1. Introduction

Cyanogenesis, the release of hydrogen cyanide (HCN) from precursors, is widely distributed in higher plants, ferns, bacteria, fungi and insects [1,2].

In plants, cyanogenic glycosides serve as a storage form of hydrogen cyanide (HCN). The generation of HCN is a two-step process which is initiated by the action of specific glycosidases followed by decomposition of the cyanohydrin (α-hydroxynitrile) into HCN and the corresponding carbonyl compound as illustrated in Fig. 1 in the case of *Linum usitatissimum*. The second step could either occur spontaneously or is enzymatically catalyzed by α-hydroxynitrile lyases (HNL) [1].

Release of gaseous HCN after plant damage is interpreted as a defense mechanism against herbivores [2]. HNLs form an interesting class of enzymes catalyzing the reverse reaction involving the stereoselective addition of HCN to

* Corresponding author. Tel.: +49-2461-613716; fax: +49-2461-612490.

[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

1381-1177/99/$ - see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S1381-1177(98)00109-X

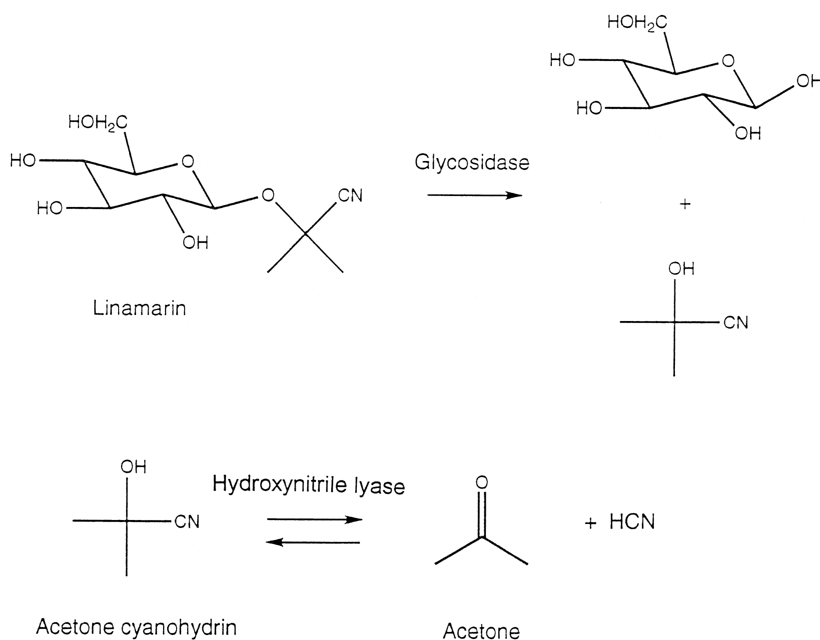

Fig. 1. The role of (R)-HNL in the catabolism of Linamarin during cyanogenesis in *Linum usitatissimum*.

aldehydes and ketones. The first enzyme catalyzed asymmetric synthesis has been reported by Rosenthaler [3] using 'emulsin', an extract from bitter almonds as a source of (R)-HNL. Due to the high functionality of the chiral cyanohydrins formed, a variety of chiral products can be derived from such intermediates by hydrolysis or reduction resulting in α-hydroxyacids and esters, α-hydroxy aldehyde, ketones and amino alcohols, etc. [4–11]. Chemical conversion of cyanohydrins with retention or inversion of the chiral center generated in the enzymatic step has been intensively studied in recent years as a possible step in the ultimate synthesis of drugs, herbicides or pesticides [12,13]. Most HNLs studied exhibit rather broad substrate specificities, accepting aromatic and/or aliphatic aldehydes or ketones as substrates [14,15].

In principle (R)- as well as (S)-cyanohydrins can be obtained from such reactions. For the development of industrial applications, the availability of the catalyst is of crucial importance, e.g., the well studied (R)-HNL from *Prunus amygdalus* is present in the seeds and can be easily obtained in large amounts [16]. Besides the enzymes in *Rosaceae*, other interesting HNLs have been isolated and characterized from plants such as *Sorghum bicolor, Hevea brasiliensis, Manihot esculenta, Phlebodeum aureum, Ximenia americana* and *L. usitatissimum* (for a review, see Ref. [15]). The enzymes appear in the tissue in rather low concentrations or only during certain developmental stages, e.g., during germination in the shoot [17–20]. To ensure a reliable and abundant supply of the enzyme (S)-HNL from *H. brasiliensis* and *M. esculenta*, the encoding genes have been cloned and expressed in microorganisms [10,21,22].

Indeed, HNLs form a heterogenous group of proteins differing in molecular mass, quaternary structure as well as glycosylation and the presence or absence of flavin adenine dinucleotide (FAD). Comparison of the DNA sequences determined so far as well as immunological studies have indicated that the HNLs in various species have evolved from different precursors

such as α/β-hydrolases, oxido-reductases or unknown proteins in a convergent process [14]. Amongst the HNLs analyzed, the structure and the reaction mechanism of the recombinant (S)-HNL from *H. brasiliensis* have been investigated in detail [23].

We were particularly interested in the (R)-HNL from *L. usitatissimum* since it accepts a variety of aliphatic ketones as substrates [24] and would thus allow the chemoenzymatic synthesis of carboxylic acids and other compounds branched at C2.

The (R)-HNL from *L. usitatissimum* has been first isolated and characterized by Xu et al. [25] and was later studied by Albrecht et al. [24]. In contrast to the (R)-HNL from *Rosaceae*, the (R)-HNL from *Linum* is not glycosylated and does not contain FAD. Therefore, the expression of active enzyme in microbial hosts appeared possible by standard techniques. This would enable us later to alter functional properties of the enzyme by protein engineering techniques. In particular, the enzyme stability at pH values < 5 has to be improved for practical applications. The native enzyme has a half-life of approximately 1 h at pH 4. As we have demonstrated previously [11,24], it is necessary to suppress the chemical reaction between HCN and carbonyl groups sufficiently to generate products with high enantiomeric excess by the enzymatic conversion. This can be achieved by lowering the pH of the reaction medium to values of 3.2–4.2 depending on the reactivity of the carbonyl group [11,26] and performing the reaction continuously in a stirred tank reactor at high enzyme concentrations and low residence times [27].

Both goals, e.g., the generation of stable enzyme variants and the availability of large amounts of catalyst require a recombinant expression system. The latter will also be used to study the reaction mechanism of a (R)-HNL by site-directed mutagenesis. Here we report an improved purification procedure of the wild-type enzyme as well as cloning and expression of the functional enzyme gene in *E. coli*.

2. Material and methods

2.1. Chemicals

Chemicals were purchased in high purity from Merck, Sigma, Fluka and Roth (Karlsruhe, D). All stock solutions were prepared with high quality water purified in a MilliQ plant and sterilized by autoclaving. With the exception of Tris all reagent solutions and lab ware used in RNA preparations were treated with 0.1% (v/v) diethylpyrocarbonate (DEPC) over night before autoclaving in order to destroy RNase. Tris buffer was prepared separately in DEPC treated glass ware.

2.2. Seeds and germination

L. usitatissimum 'Futter Leinsamen' was purchased from local sources (Landhandel Irnich, Frechen-Königsdorf, D) 130 g seeds were soaked for 2 h in water, collected and placed on moist pulp sheets in a plant box for germination under controlled light conditions. A 60 W plant light (Paulmann) was operated in light (14 h)–dark (10 h) cycles. After 10–14 days, hulls were removed from the seedlings and primary leaves were collected and stored frozen at −20°C.

2.3. Enzyme extraction

Before cell disintegration the plant tissue was placed in liquid nitrogen and the brittle material powderized by mortar and pestle. The powder was suspended (15% w/v) in 20 mM potassium phosphate (Kpi) buffer, pH 6.2, and kept for 20 h at 4°C. Then Triton X 114 (Fluka, Neu-Ulm, D) was added to a final concentration of 2.5% (w/v) and the suspension was gently mixed at room temperature for 2 h before heating to 30°C for 30 min. The heating step serves to separate a Triton X 114 rich bottom phase and a Triton poor top phase containing the enzyme. The phases were separated by centrifugation (Sorvall RC 5B, 20 min, 4000 rpm, GSA rotor, Du Pont,

Bad Homburg, D) and the top phase was collected by decantation. Any remaining solid material was removed with the bottom phase during the centrifugation.

2.4. Hydrophobic interaction chromatography

Adding finely ground ammonium sulfate with stirring, the protein solution was brought to 40% saturation and any precipitated protein was removed by centrifugation (Sorvall RC 5B, 20 min, 10,000 rpm, GSA rotor).

The supernatant was applied to a column (30 ml) packed with butyl sepharose FF (Pharmacia Biotech, Freiburg, D) and equilibrated against 20 mM Kpi buffer, pH 6.2 containing 40% ammonium sulfate. The column was washed with equilibration buffer until the adsorbance at 280 nm of the eluate returned to base line values. Elution was carried out by a step change to 16% ammonium sulfate in the buffer. The enzyme-containing fraction was desalted by gelfiltration using a G25 Sephadex column (Pharmacia, Biotech) with 20 mM Kpi buffer, pH 6.2, as an eluent.

2.5. Immobilized metal affinity chromatography

A column was filled with 50 ml chelating sepharose FF (Pharmacia Biotech) and equilibrated with 20 mM $CuSO_4$ followed by washing with 20 mM Kpi buffer, pH 6.2, until the eluent was free of Cu^{++} ions.

The (*R*)-HNL was applied to the column and washed consecutively with 20 mM Kpi buffer, pH 6.2, and 50 mM sodium acetate, pH 4.5, until the adsorbance of the eluate at 280 nm approached base-line values. (*R*)-HNL was finally eluted using a linear gradient from 0–50 mM imidazol in 20 mM Kpi buffer, pH 6.2.

2.6. Preparative gel electrophoresis

To isolate homogeneous protein, gel electrophoresis was carried out under denaturing conditions according the method of Goldberg [28]. For the preparation of the separating gel, 24 g urea were dissolved in 50 ml of a 0.375 M Tris buffer, pH 8.8, containing 7.5% acrylamide. Polymerization was started by adding 120 μl of a 10% aqueous ammonium peroxodisulfate and 40 μl (N,N,N',N'-Tetramethylethylendiamine) TEMED. To prepare the collecting gel, 12 g urea were dissolved in 25 ml 0.125 M Tris buffer, pH 6.8, containing 4.5% acrylamide. A flat bed electrophoresis chamber was used for electrophoresis (BioRad, Munich, D).

The protein sample (1.0 ml) was mixed with 1 g urea, 0.1 ml β-mercaptoethanol, and 0.2 ml 0.125 M Tris buffer, pH 6.8, containing 10% glycerol and 0.1% bromophenol blue as a tracer. The sample was placed in the chamber and electrophoresis started at a constant current of 10 mA. 25 mM Tris with 0.129 M glycine buffer employed as the electrophoresis buffer.

When the tracer migrated from the separation gel into the buffer chamber, the electrophoresis was stopped and the gel was transferred into a dish for staining with Coomassie blue according to Merril [29]. The separated bands were cut from the gel under illumination. The (*R*)-HNL containing band was identified by N-terminal sequencing upon blotting onto a polyvinyldifluoridene (PVDF) membrane (IMMOBILON-P, Schleicher and Schüll, Dassel, D). For N-terminal sequencing an automated Edman degradation using a liquid phase sequencer (Model 477 A) equipped with an on-line HPLC unit (120 A) from Applied Biosystems (Weiterstadt, D) was used. Further, pieces of the separated gel bands were subjected to PAGE according to Laemmli [30] and restained with silver nitrate as described by Rabilloud [31].

2.7. (R)-HNL assay

The activity was measured as described by Albrecht et al. [24] using acetone cyanohydrin as a substrate. The HCN liberated during 10 min of reaction was quantified using a commercial kit 'Spectroquant 14.800 cyanid' from

Merck (Darmstadt, D) and following the instructions of the manufacturer. The kit is based on a modified König reaction [32] and yields a polymethine dye. A linear calibration curve $OD_{585} = 0.703 \cdot [CN]$ ($r^2 = 0.999$) was obtained in the range of 0–2.5 mM using lithium cyanide for calibration.

2.8. Production of polyclonal antibodies

For immunization purposes, pure denatured (R)-HNL was prepared as described above. The appropriate band was destained using 40% ethanol solution containing 10% glacial acetic acid and subjected to sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was detected after separation, rinsing the gel with ice cold demineralized water and placing it in a pre-cooled dish containing 0.25 M KCl with 1 mM dithiothreitol (DTT). After about 5 min gentle agitation at 0°C, the protein band was visible as a white precipitate against a black background. The identified band was cut out from the gel and the protein concentration was estimated by SDS-PAGE using BSA (Fluka) for calibration. A piece of gel containing 400 μg (R)-HNL was placed in a vessel and supplemented to 4 ml with adjuvant (Sigma, Munich, D). A total of 2.7 ml of this mixture were used for the first immunization of a rabbit.

The remaining protein was again diluted with 1 ml adjuvant and injected after 6 weeks to boost the production of IgGs. Fifteen weeks after the booster injection the animal was killed and the blood was collected and cooled to 4°C. Blood cells were separated by centrifugation (Sorval/RC5B, 10,000 rpm, 15 min, SS34 rotor) and the serum obtained stored in aliquots at −20°C.

2.9. Westernblotting and immuno-staining of immobilized proteins

Proteins were transferred to PVDF- and nitrocellulose membranes (OPTITRAN BA-S 83, Schleicher and Schüll, Dassel, D) as described by Kyhse-Andersen [33] using a Novablot 2117 blotting chamber (Pharmacia Biotech). The immunoblots were incubated over night at 4°C in blocking solution (10 mM Tris–HCl, pH 7.5, 5% milk powder, 1% Tween 20) followed by incubation with polyclonal antibodies against *Linum* (R)-HNL for 1 h at 37°C in TBS-T (Tris-buffered saline (10 mM Tris–HCl, pH 7.5; 0.9% NaCl) containing 0.2% BSA and 0.1% Tween 20). Subsequently the blot was washed 3 times for 20 min in TBS-T. The secondary antibody conjugated with horseradish peroxidase (Boehringer Mannheim, Mannheim, D) was diluted 1:10,000 in TBS-T and the membrane incubated 1 h at 37°C. After washing the membrane 3 times for 20 min in TBS-T, bound peroxidase was detected using enhanced chemiluminescence (ECL)-detection solution (Amersham–Buchler, Braunschweig, D) on a Luminograph (Berthold, Bad Wildbad, D).

2.10. Refolding of denatured (R)-HNL

Recombinant (R)-HNL was concentrated with Centricon spin columns (Amicon) and denatured with 8 M urea, DTT and EDTA as described by Rudolph [34]. After removal of the EDTA on a PD10 gel filtration column (Pharmacia Biotech), the protein was rapidly diluted 1:10 in renaturing buffer (10 mM Kpi buffer containing no Zn^{++}, 2 μM Zn^{++} or 1 mM Zn^{++}, respectively). Additionally, denatured protein was diluted 1:10 in Kpi buffer containing 3 mM reduced glutathione and 0.3 mM oxidized glutathione [34] in the presence or absence of 2 mM Zn^{++}, respectively. HNL activity was measured after incubation periods of 1 h and 20 h at room temperature, respectively.

2.11. Molecular biology techniques

The following kits and enzymes were purchased and used following the manuals of the manufacture's:

Nucleo spin extract kit (Macherey and Nagel, Düren, D).

3′/5′-RACE kit (Boehringer Mannheim).
pMos blue T-vector kit (Amersham–Buchler).
Restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I) as well as RNaseA and T4 DNA-ligase were purchased either from Boehringer Mannheim, Pharmacia Biotech or MBI Fermentas (Vilnius, Lithuania).
M-MuLV reverse transcriptase, *pfu*-DNA polymerase, and RNase Block I were obtained from Statagene (Heidelberg, D), *Taq*-DNA polymerase from BioMaster (Cologne, D) and terminal transferase from MBI Fermentas. *Pfu*-DNA-polymerase was obtained from Stratagene (Heidelberg, D).

The different *E. coli* strains used for cloning and expression were obtained from Boehringer Mannheim (SG 13009[pREP 4]), Pharmacia Biotech (JM 105), Stratagene (XL1 Blue) and Novagen (Madison/USA) (AD494 (DE3)).

The following commercial vectors were employed:

pKK 223-2	Pharmacia Biotech, Freiburg, D
pQE60	Qiagen, Hilden, D
pQE9	Qiagen, Hilden, D
pMos Blue	Amersham–Buchler, D
pUC18	Pharmacia Biotech, Freiburg, D
pBTac2	Boehringer Mannheim, Penzberg, D

2.12. PCR-primers

The primers used were ordered at MWG Biotech, München, D or synthesized using a DNA-synthesizer model 391 PCR-MATE (Applied Biosystems, Weiterstadt, D). Lyophilized primers were dissolved in sterile water, adjusted to 100 pmol/μl and stored frozen at −20°C.

The following primers were employed:

#1465	5′-CGCTTAAGACTGACGTCAA-TGGCTG − 3′
#1466	5′-TGACGTCAATGGCTGAGAG-CTCATG − 3′
#1488	5′-CGCTTAAGACTGACGTCAA-TGGCTGAGA GCTCATGTTTT-TTTTTTTTTTTTTT − 3′
luHydN1	5′-AT(ACT)AC(ACGT)TG(CT)AA-(AG)G C(ACGT)AT(ACT)ATGCT − 3′
luHydN2	5′-ATGCT(ACGT)AA(AG)GA(A-G)GC(ACGT)AA(AG)CT − 3′
luHydN3	5′-ACAATGGCGTCTCTTCCTG − 3′
luHydN4	5′-CGATAGAATTCAATGGCGT-CTCTTCCTGTTA − 3′
luHydN5	5′ − CTGCTGTGATTGCTGCC-AAAGAGC − 3′
luHydN7	5′-AGTTCAGGATCCGCGTCTCT-TCCTGTTAGCT − 3′
luHydC2	5′-GAATCAATAATCGTTCAAC-TTG − 3′
luHydC3	5′-ACTGCAAGCTTGAATCAA-TAATCGTTCAACTTG − 3′
luHydC4	5′-ATGCTCTCGATTATCCCAAC-A − 3′
luHydC5	5′-ATTATCCCAACACCTTCATG-C − 3′
luHydC6	5′-GGTGGCCCCCAGTTCCATG -GCCAT − 3′
luHydC7	5′-ACCGGATCCATAATCGTTC-AACTTGATCACG − 3′

2.13. Gel electrophoresis of double stranded linear DNA

Electrophoresis was performed in agarose gels (0.5–2% w/v) according to Aaij and Borst [35] and Helling et al. [36] in 40 mM Tris/acetate buffer containing 1 mM EDTA. A 100 μg ethidium bromide was added to aliquots of 70 ml agarose solution for gel preparation.

Electrophoresis was performed at constant currents in the range of 50–70 mA. For size calibration of fragments the 1 kb DNA ladder from Gibco (Eggenstein, D) was used. Evaluation and documentation of the gels was carried out with an Eagle Eye II system (Stratagene, Heidelberg, D).

2.14. Gel electrophoresis of RNA

For size-separation of RNA fragments formaldehyde containing gels were prepared ac-

cording to Lehrach et al. [37] and fragments were separated at constant current of 60 mA in 3-(N-morpholino)ethane sulfonic acid (MOPS)–buffer. For calibration the 0.24 to 9.4 kb RNA ladder from Gibco was used.

2.15. Isolation of plasmid DNA

Plasmid DNA was prepared from recombinant cells using the Quantum Prep Kit from Bio Rad according to the instructions given. Additionally, lysis under alkaline conditions was performed as described by Birnboim and Doly [38]. To obtain DNA for sequencing from *E. coli* strains JM 105 or SG 13009 an additional protein extraction step was carried out using 500 μl of a solvent mixture of phenol:chloroform: isoamyl alcohol (25:24:1) to avoid interference by restriction enzymes present in the host.

2.16. Isolation of DNA fragments

DNA bands of interest were cut from agarose gels and purified using the Nucleo spin extract kit according to the manufacturer's instructions. The amount and purity of the recovered DNA was routinely checked by electrophoresis of an aliquot on an agarose gel. Transfer of double stranded DNA from agarose onto Nylon-66 membranes NY 45 (Serva, Heidelberg, D) was accomplished by the method of Southern [39]. Nucleic acids were fixed by UV treatment (240 nm) in a strata linker (Stratagene).

2.17. cDNA-synthesis

Poly(A)$^+$-RNA was extracted from total-RNA solutions with Oligotex-dT (Qiagen) following the instructions from the manufacturer. cDNA was synthesized using 2 ng #1488 as cDNA-primer and 50 U M-MuLV reverse transcriptase. After incubation for 1 h at 37°C the cDNA-solution was diluted with 0.1 × STE (1 mM Tris–HCl pH 7.5, 10 mM NaCl, 0.1 mM EDTA) and applied onto a 0.5 ml Centricon 30 spin column (Amicon, Beverly, USA). Small molecules were removed by centrifugation (10 min, 10,000 rpm, Model 5415C, Eppendorf, Cologne, D). The cDNA was washed 3 times by subsequent addition of 0.5 ml 0.1 × STE followed by centrifugation, prior to removal from the column and stored at −20°C.

2.18. '3 / 5'-rapid amplification of cDNA ends (RACE)

For cloning the 3′-fragment of the (*R*)-HNL mRNA, 3′-RACE was used as described by Frohman et al. [40]. cDNA-synthesis was primed with #1488 and used as template for the first PCR with luHydN1 as HNL-specific and #1465 as cDNA-specific PCR-primer. An aliquot of this reaction was used as template for a second PCR with luHydN2 and primer #1466.

5′-RACE was performed using the 3′/5′-RACE kit from Boehringer Mannheim according to the prescribed instructions. luHydC2 was used as cDNA-primer, luHydC4 and LuHydC5 were employed as HNL-specific PCR-primers [41].

2.19. Polymerase chain reaction

The open reading frame of the HNL-gene was amplified from cDNA using 25 pmol of each PCR-primer and *Taq*- or *Pfu*-polymerase. Different PCR-primers were used to generate recognition sites for restriction endonucleases which allowed directional cloning of the PCR-fragments after digestion with the appropriate enzymes.

Before the first cycle, the reaction mixture was overlayed with 100 μl paraffin oil (Sigma) and incubated for 5 min at 94°C. PCR was performed with a Robocycler Gradient 40 (Stratagene). After 30–35 cycles, the reaction mixture was incubated for 10 min at 72°C, the upper oily phase removed and proteins extracted with phenol:chloroform:isoamyl alcohol (25:24:1).

2.20. C- and N-terminal fusion of a hexahistidine tag (His-tag)

For adding a C-terminal His-tag, PCR was carried out as described above, using luHydN4 and luHydC7 as PCR-primers to introduce an *Eco*RI-site before the Start-codon and a *Bam*HI-site instead of the Stop-codon. PCR-fragments were co-digested for 2 h at 37°C with *Eco*RI and *Bam*HI. The fragment was isolated by preparative agarose gel electrophoresis and ligated into the similarly treated pQE60 using T4 DNA ligase.

An N-terminal His-tag was generated using luHydN7 and luHydC3 as PCR-primers. luHydN7 inserts an *Eco*RI-site instead of the Start-codon, while luHydC3 generates a *Hin*dIII-site behind the Stop-codon. The *Eco*RI/*Hin*dIII-digested PCR-fragment was isolated and ligated into *Eco*RI/*Hin*dIII-digested pQE9 as expression vector. For DNA-sequencing the construct was ligated into pUC18 and both strands were sequenced using an A.L.F.-DNA-sequencer (Pharmacia Biotech).

The sequence was deposited in GenBank under accession number AF024588.

2.21. Expression and purification of recombinant (R)-HNL

E. coli strain AD494(DE) containing plasmid pQE9-HNL was grown in 2 L of Luria–Bertani (LB) medium, pH 7.5, supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin, to the log-phase at 28°C. Protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) over 4 h.

Cells were harvested by centrifugation and suspended in 20 ml of lysis buffer (50 mM Kpi, 5 mM EDTA, pH 7.5). The cell suspension was lysed by sonication with a pulsed sonifier (Bandelin Sonoplus HD60) for 2×30 s with 25% intensity on ice, after treatment with 10 mg/ml lysozyme for 30 min.

The lysate was centrifuged to pellet cell debris and insoluble fractions (inclusion bodies). The supernatant contained soluble (*R*)-HNL.

The crude extract was loaded onto a Ni–nitrilotriacetic acid (NTA) column, equilibrated with 50 mM Kpi-buffer, pH 7.5. The column was washed with the binding buffer by adding imidazole at a 10 mM concentration to eliminate proteins with endogenous histidine residues. The hexa-histidine-tagged (*R*)-HNL was eluted by a step gradient at an imidazole concentration of 200 mM in 50 mM Kpi-buffer, pH 7.5.

Column fractions were analyzed on 10% SDS-PAGE gels subjected to silver-staining.

3. Results and discussion

3.1. Protein purification and production of antibodies

The period of enzyme solubilisation from the powdered plant tissue was prolonged following unpublished observations from G. Goetz [42] which indicated higher enzyme yields after overnight incubation at 4°C. During this period, remaining cyanogenic glycosides are decomposed leading to very high background values in the enzyme assay in the absence of added substrate. Therefore, the activities in the crude extract could not be determined accurately. The extraction procedure described using Triton X 114 makes use of the temperature dependent hydration of ether groups in the ethylene oxide tail of the nonionic detergent as described by Bordier [43] and Kehren [44]. The step serves to remove hydrophobic proteins and especially membrane bound chlorophyll which partition preferentially to the detergent rich bottom phase [45,46], while (*R*)-HNL remains in the nearly colorless detergent depleted top phase. After adjusting the protein solution to 40% saturation with ammonium sulfate, (*R*)-HNL could be adsorbed onto butyl sepharose FF and was washed free of residual Triton X 114. The enzyme was eluted by a step change to 16% saturation with ammonium sulfate and yielded a specific activity of 6.1 U/mg, which is 20 times higher compared to the specific activity in the crude

extract reported by Albrecht et al. [24]. The metal chelating step was introduced because the (*S*)-HNL from *Sorgum* and also (*R*)-HNL from *Prunus* could be highly purified by this method [47,48]. With (*R*)-HNL from *L. usitatissimum*, a two fold purification could be achieved, resulting in a catalyst preparation containing ≈ 25% (*R*)-HNL.

The presently used *Linum* variety contains an unknown protein of 42 kD, which could not

```
aaactcatag aacacacaga gtttacagag tttttttcaa gagttcaaca    50
M  A  S  L   P  V  S   F  A  K   P  D  K  N   G  V  I
atggcgtctc ttcctgttag ctttgctaaa cccgacaaga atggagtcat   100
 T  C  K   A  I  M  L   K  E  A   K  L  P   G  M  S  Y
cacttgcaag gcgattatgc tgaaggaggc gaagttgcca ggaatgtcat   150
 A  D  T   V  Q  I   I  D  I  Q   V  D  P   P  Q  N
acgctgatac cgtccagata atagacatcc aagtggaccc gccgcaaaat   200
V  E  L  R   V  K  M   L  C  A   S  V  C  R   T  D  I
gtcgagctta gggttaagat gttgtgtgca agtgtgtgcc gcaccgatat   250
 L  T  I   E  G  F  M   A  P  T   Q  F  P   K  I  N  G
tttaaccatt gaaggcttca tggccccgac tcaattccct aaaatcaatg   300
 H  E  G   V  G  I   I  E  S  M   G  P  D   T  K  N
ggcatgaagg tgttgggata atcgagagca tgggcccgga cacgaagaac   350
F  K  V  G   D  V  I   V  A  P   T  L  G  E   C  Q  T
ttcaaagtgg gtgacgtcat cgtggctcca acgttaggag agtgccagac   400
 C  S  S   C  R  S  G   R  T  N   F  C  Q   N  Y  G  A
ctgcagcagc tgcaggtccg gccgaaccaa cttctgccag aactacgggg   450
 N  E  S   A  L  E   P  D  G  T   S  R  F   S  Y  I
ccaatgaatc ggcgctcgaa ccggacggta cctcgagatt ctcgtacatc   500
D  S  D  G   K  K  K   L  L  Y   Y  K  L  G   C  S  T
gattcggacg gtaagaagaa gctcctttac tacaagctgg gatgctccac   550
 W  T  Q   Y  M  V  V   D  S  N   Y  A  T   K  L  N  E
gtggacgcag tacatggtgg tcgactccaa ctacgccacc aagctcaacg   600
 I  A  P   E  L  P   P  P  H  G   S  I  L   S  C  A
agatcgcacc ggagctccct ccgccacatg gcagcatcct ctcatgtgct   650
F  A  T  G   Y  G  A   V  W  L   D  A  A  V   Q  E  G
tttgccactg gatacggtgc tgtgtggctc gacgccgccg tccaggaagg   700
 D  S  V   A  I  F  G   V  G  S   V  G  I   S  A  V  I
cgactccgtc gccatttttg gagtcggctc cgtcggcata tctgctgtga   750
 A  A  K   E  L  K   A  K  Q  I   I  V  V   D  R  N
ttgctgccaa agagctgaaa gcgaagcaga taatagtggt ggacaggaac   800
E  Y  K  L   K  M  A   M  E  L   G  A  T  H   C  I  N
gagtacaagc tgaaaatggc catggaactg gggccaccc actgcatcaa    850
 S  E  K   L  P  E  G   V  T  P   S  Q  A   V  R  K  L
ctccgagaaa ctacccgagg gggtcacccc ttcgcaagcg gttaggaaac   900
 T  P  K   E  V  G   V  D  A  S   I  E  S   S  G  Y
tcaccccaa  ggaagtcgga gtcgatgcga gcatcgaatc ctcgggctac   950
D  V  F  M   N  E  A   M  K  A   A  I  H  G   K  A  K
gacgtcttca tgaacgaagc catgaaagcc gccatccacg ggaaagccaa  1000
 T  V  I   T  G  E  G   I  Y  E   N  D  R   I  F  F  D
gaccgtgatt accggagaag gaatttacga aaacgaccga atcttcttcg  1050
 F  K  D   F  L  F   G  G  N  V   V  G  N   V  T  G
atttcaagga cttcctgttc ggcgggaacg tagtcggaaa cgtcacgggt  1100
R  V  R  I   H  S  D   F  P  G   L  L  R  K   A  Q  E
cgggttagaa tccatagcga tttcccaggg ttgctgagaa aggctcaaga  1150
 P  V  I   R  A  G  M   D  K  I   L  G  Y   D  A  A  T
accggtaatc agagctggaa tggataaaat cttggggtac gatgcggcaa  1200
 M  K  C   K  Y  E   V  D  I  R   E  G  T   P  A  L
ctatgaagtg caagtacgag gtcgacattc gtgagggtac tcctgcatta  1250
L  K  A  L   E  E  V   E  N  V   D  C  V  K   L  V  I
ctgaaagcat tggaagaggt ggagaatgtg gattgcgtca aactcgtgat  1300
 K  L  N   D  Y  *
caagttgaac gattattgat tcggtttacc gaatttgatt tcgtaaatcg  1350
attctgcccc ggctatatgt aacttctaaa taatcacgat cctattcgtg  1400
agtgtgtgtg tgtttcggtt attttattta tttcaatttg aataaagaaa  1450
ctgtggtcta tcaaatacaa ctgatagatc acaagagttt ctatgtatga  1500
agttgttgct gtttctttca ataaaaatct atgcatttgg ctttgaaaaa  1550
aaaaaaaaaa aaaaaaaaaa aaa
```

Fig. 2. Nucleotide sequence and derived amino acid sequence from *Linum* (*R*)-HNL. The stop codon is marked with an asterisk (*). A putative polyadenylation signal at position 1441 is underlined. The independently derived amino acid sequence data are printed in italics (including data obtained from Albrecht et al. [24]). Sequence data obtained in this study are marked in grey.

be separated effectively from (*R*)-HNL by ion exchange, gel-filtration or SDS-PAGE as described by Albrecht et al. [24]. To obtain pure protein for immunization we had to resort to preparative slab gel electrophoresis in the presence of urea. Since the N-terminal sequence of the (*R*)-HNL was known from our previous studies, the protein band could be unequivocally identified. The N-terminal protein sequence found is shown in Fig. 2 and corresponds to the shortest isoform reported by Albrecht et al. [24]. The longer sequences were not observed, which may be a consequence of the longer initial incubation time during the extraction of the powdered plant tissue. Starting from 120 g of plant tissue 250 μg pure protein were obtained.

Immunization was carried out in a rabbit. Before immunization, the animal was tested with respect to antibodies directed against proteins from *Linum* and *E. coli* XL1 Blue using Western blot analysis. 78 ml of rabbit serum were obtained 21 weeks after the first immunization, and provided detection of less than 10 ng (*R*)-HNL specifically in crude extracts of *Linum* or *E. coli* after 1:50,000 dilution.

3.2. Cloning the (R)-HNL gene from L. usitatissimum

To clone the desired plant gene in *E. coli* we decided to utilize reverse transcriptase polymerase chain reaction (RT-PCR) techniques. Since the known protein sequence did not allow the design of two primers of sufficient length to be of sufficient distance apart, 3′-RACE-PCR ([40,41]) was carried out using the anchor primer #1488 for the cDNA synthesis by the M-MuLV reverse transcriptase, the gene specific primers luHydN1 and luHydN2 and the cDNA-specific primers #1465 and #1466 for the subsequent amplifications of the gene fragment.

A single band of 1500 bp, probably corresponding to the (*R*)-HNL gene was observed (Fig. 3) which was ligated into pMos Blue. The resulting plasmid was transformed in *E. coli* XL1 Blue.

Colonies were grown on Luria–Bertani medium containing ampicilline (25 μg/ml) (LB^{amp}) agar plates and checked for the presence of the 1500 bp insert. The PCR fragment obtained was partially sequenced. Results from

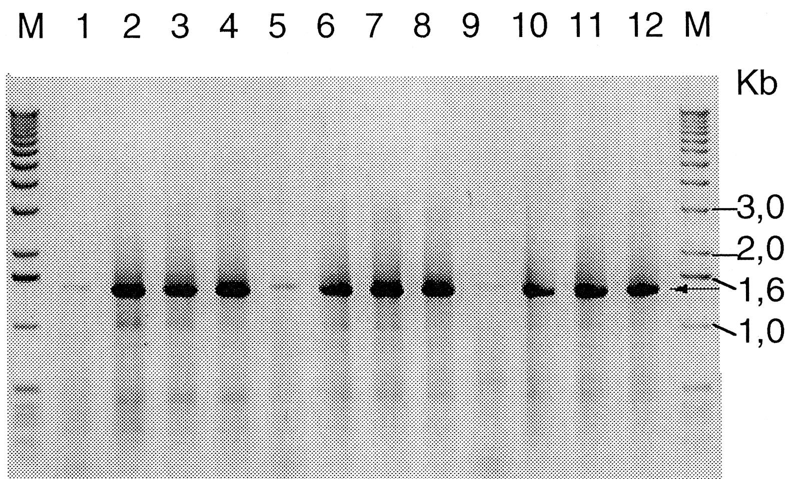

Fig. 3. Results of 3′-RACE analyzed on a 0.8% agarose gel. **Lanes 1,5,9** display the results of the first round of PCR at annealing-temperatures of 54°C, 57°C and 60°C. **Lanes 2–4, 6–8** and **10–12** display the second PCR using an aliquot of the first PCR as template. The annealing temperatures were 54°C, 57°C and 60°C. Details are given in Section 2.

the 3′-end of the amplified cDNA confirmed the expected sequence according to the known N-terminal sequence of the protein.

To generate the complete gene, a 5′-RACE was carried out ([49]). For the cDNA synthesis, luHydC2 was used as a primer. The cDNA was poly-adenylated and two consecutive amplifications were carried out, using luHydC4 and luHydC5 as gene specific primers. A specific 500 bp fragment was observed after the second PCR reaction and was subsequently cloned and sequenced. The results revealed that the band contained the expected sequences corresponding to the data from protein and DNA analysis of the 1500 bp fragment.

To isolate the complete coding sequence for the (R)-HNL, RT-PCR was carried out once more with luHydN3 and luHydC2 as specific primers and *Pfu*-DNA-polymerase. A 1300 bp PCR fragment was obtained and ligated into pUC18. After transformation of *E. coli* XL1 Blue with the vector and selection on LB^{amp} plates, colonies were checked for the presence of the 1300 bp insert. One positive clone was selected and the DNA of both strands was sequenced. Besides the vector-derived universal and reversal primers, the gene specific primers luHydN5 and luHydC6 were employed. The results are summarized in Fig. 2.

An open reading frame of 1266 bp was found (Position 51 to 1315) corresponding to 422 amino acids. The gene sequence reveals that the full length enzyme is 8 amino acids longer at the N-terminus than the longest protein sequence observed [24] so far in the (R)-HNL isolated from *Linum* seedlings (Fig. 2). It remains unclear whether the observed processing of the N-terminal region in the plant is an artefact of the (R)-HNL extraction procedure (see above). Since active enzyme was expressed in full length clones and also from constructs carrying an N-terminal extension (His-tag, see below), the N-terminal processing obviously is not needed to activate the enzyme. A comparison with the DNA sequence independently derived by Trummler and Wajant [50] using a different cloning approach shows 7 base exchanges in the coding region, 5 of which do not alter the protein sequence. The two adjacent base exchanges in position 399 and 400 lead to a change from valine to threonine, corresponding to position 117 of the amino acid sequence. This could be verified by sequencing an independently derived clone of the HNL-gene. The

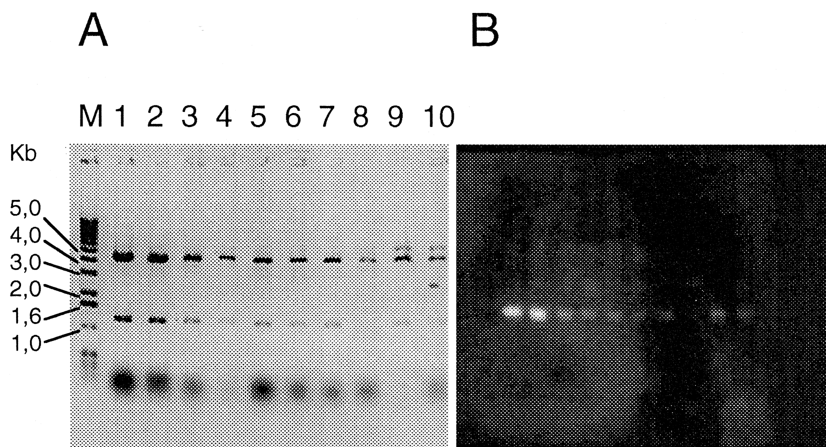

Fig. 4. Southern blot analysis of recombinant pBTac2 plasmids. **A** displays analysis of *Eco*RI/*Hin*dIII-digests of plasmids derived from different clones on agarose gels. **B** shows the detected signals after Southern blotting and hybridization with a DIG-labelled PCR-fragment from the (R)-HNL-gene.

Table 1
HNL-acitivity in *E. coli* JM105/pBTac2 crude extracts at various fermentation temperatures

Temperature	Total activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)
18°C	0.8	1.2	0.7
25°C	2.5	1.95	1.3
30°C	2.4	1.8	1.4
37°C	0.15	1.9	0.01

A total of 100 ml LB^{amp} cultures were grown 2 h at 37°C and cooled down for 1 h at the appropriate temperature before protein synthesis was induced with 1 mM IPTG. After fermentation over night, the cells were harvested and disrupted enzymatically.

small differences detected may reflect different variants of the plant.

3.3. Expression of (R)-HNL

To express the recombinant enzyme in *E. coli,* the gene was cloned into the expression plasmids pBTac2 and pKK 223–2. The primers luHydN4 and luHydC3 were used to introduce an *Eco*RI and *Hin*dIII cleavage site adjacent to the start and stop codon, respectively. Both plasmids carry a β-lactamase gene to select transformants. Fig. 4 shows the Southern blot analysis of the expression plasmids generated. In addition, a hexa-histidine coding sequence was introduced in frame at the N- as well as the C-terminal position to facilitate purification of the enzyme. The expression plasmids were transformed in different *E. coli* strains as a host.

Table 1 summarizes results obtained during growth of *E. coli* JM105 carrying pBTac2 at different temperatures. At 37°C, the activity is barely detectable in the crude extract, while Western blots showed a high concentration of protein in the pellet (data not shown). At 30°C and lower temperatures, active enzyme is found in the soluble fraction of the crude extract, but there are still significant amounts in the pellet after cell disintegration. As shown in Figs. 2 and 7 (*R*)-HNL contains 12 cysteine residues, which may contribute to the formation of inclusion bodies during expression in *E. coli*. Additionally the requirement for intramolecular disulfide bonds in (*R*)-HNL could not be excluded [51]. Therefore, the *E. coli* strain AD494(DE3) was tested as a host, which is deficient in thioreductase activity.

As shown in Table 2 and Fig. 5, the highest activity of soluble (*R*)-HNL was found using the thioreductase deficient host. The Western blot analysis shows that in all the gene constructs and plasmid/host combinations, large amounts of (*R*)-HNL are found as insoluble protein aggregates (Fig. 5). The ratio between soluble and aggregated material appears best using AD494(DE) as a host when approximately equal amounts of soluble and aggregated enzyme were obtained. The data also demonstrate that adding a His-tag at the C-terminus leads to inactive protein, while elongation of the protein chain at the N-terminus yields active enzyme (Table 2). Assuming that the three dimensional structure of (*R*)-HNL is related to the well known horse liver alcohol dehydrogenase

Table 2
HNL-activity obtained with various expression plasmids and host cells

E. coli-strain	Plasmid	Modification	Total activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)
JM105	pBTac2-HNL	none	2.0	5.7	0.35
JM105	pQE60-HNL	C-term. His-tag	0.0	6.6	0.0
JM105	pQE9-HNL	N-term. His-tag	0.5	1.4	0.35
SG13009	pQE9-HNL	N-term. His-tag	1.3	2.6	0.5
AD494(DE3)	pQE9-HNL	N-term. His-tag	2.5	2.3	1.1

A total of 400 ml LB^{amp} cultures were grown for 2 h at 30°C before protein synthesis was induced by adding IPTG to 0.5 mM end concentration. After fermentation for 16 h at 30°C the cells were harvested and disrupted mechanically.

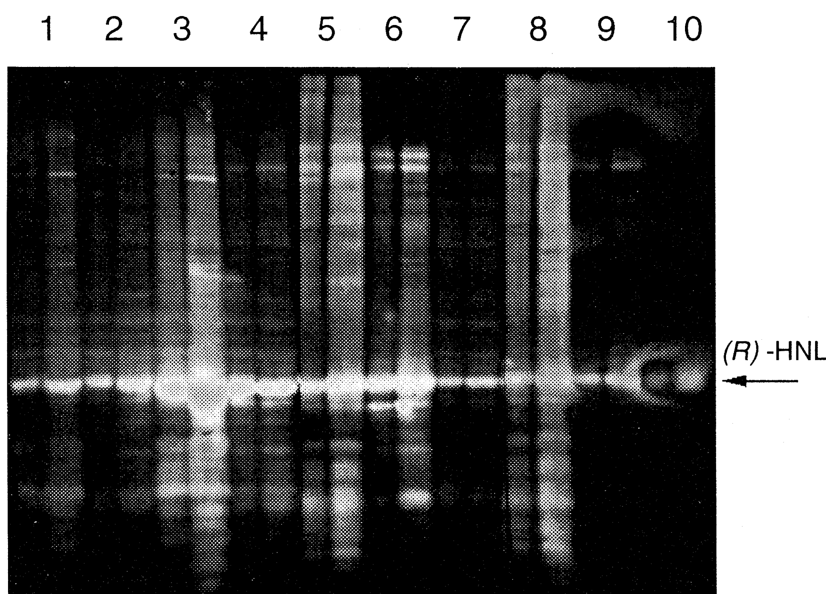

Fig. 5. Westernblot analysis of different recombinant (*R*)-HNL (indicated with an arrow) producing *E. coli* strains. **Lane 1**: pellet of *E. coli* JM105 crude extract, (*R*)-HNL with C-terminal His-tag; **lane 2**: supernatant of the *E. coli* JM105 crude extract (lane 1); **lane 3**: pellet of *E. coli* SG13009 crude extract; **lane 4**: supernatant of the *E. coli* SG13009 crude extract; **lane 5**: pellet of *E. coli* AD494(DE3), (*R*)-HNL with N-terminal His-tag; **lane 6**: supernatant of the *E. coli* AD494(DE3) crude extract; **lane 7**: supernatant of non-induced *E. coli* SG13009; **lane 8**: pellet of *E. coli* SG13009 crude extract, (*R*)-HNL with N-terminal His-tag; **lane 9**: supernatant of the same crude extract as in lane 8; **lane 10**: crude extract of *L. usitatissimum.*

(ADH) [52] or $\beta_1 \beta_1$-human ADH [53], the N- and C-terminus of the protein was expected to be exposed on the surface. Nevertheless, only the N-terminal His-tag yielded an active (*R*)-HNL. The sequence of (*R*)-HNL is about 18 amino acids longer at the C-terminus than the ADHs and its integration into the protein structure is not known presently.

The recombinant (*R*)-HNL with the N-terminal His-tag was produced in shake flasks and isolated from the soluble and insoluble fraction using a Ni^{++}-NTA superflow column [54]. The N-terminal protein sequence was verified by Edman degradation, and gave the expected sequence **MRGS (H_6) GS *ASCP***. The underlined amino acids correspond to the authentic sequence downstream of the original start codon for methionine (Fig. 2). The purified enzyme was found to have a specific activity of about 76 U/mg (Table 3). For the native enzyme a specific activity of 53 U/mg has been reported by Albrecht et al. [24]. The differences originate from different purities of the preparations. However, Trummler and Wajant obtained a specific

Table 3
Purification of recombinant (*R*)-HNL with N-terminal His-tag from *E. coli* AD494(DE3) crude extracts was chromatographed on a Ni–NTA column

	Volume (ml)	Total activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)
Crude extract	14	137.2	9.4	1.04
Ni-NTA-column	24.5	42.1	0.07	75.7

The data refer to cells derived from a 2-l culture.

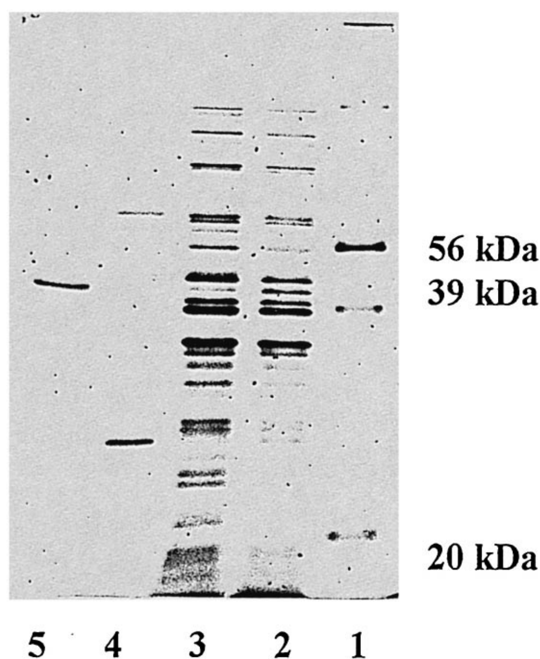

Fig. 6. SDS-PAGE analysis of different purification steps of recombinant *Linum* (R)-HNL expressed in *E. coli* AD494(DE3). **Lane 1**: Marker (56 kDa, 39 kDa, 20 kDa); **lane 2**: crude extract; **lane 3**: Ni-NTA-chromatography: unbound fraction; **lane 4**: Ni-NTA-chromatography: fraction eluted with 20 mM imidazole; **lane 5**: (R)-HNL fraction eluted with 200 mM imidazole. The proteins were visualized by silver staining.

activity of 40 U/mg for a His-tagged (R)-HNL from *Linum* using different assay conditions [50].

The expression system pQE9/*E. coli* AD494 (DE3) can be utilized to produce active (R)-HNL, which can be purified to homogeneity in one step on a Ni-NTA column to (Table 3, Fig. 6). Under unoptimized fermentation conditions, about 70 U soluble enzyme are obtained per liter culture volume, which is equivalent to 800-1000 g plant tissue as starting material. The expression of the active (R)-HNL carrying a N-terminal His-tag paves the way for the isolation of mutant HNL with improved stability.

Table 4
Sequence similarity search with (R)-HNL using WU-blastp [58]

Enzyme	Organism	High score
ADH I	potato	611
FADH (ADH III)	*Candida maltosa*	507
FADH (ADH III)	*Synechocystis* sp.	504
ADH III	*Escherichia coli*	483
ADH	*Caenorhabditis elegans*	478
ADH III	rabbit	466
FADH/ADH III	atlantic hagfish	461
FADH/ADH III	rat	458
FADH/ADH III	*Saccharomyces ceresisiae*	458

Nevertheless, significant amounts of the expressed protein are still found in the insoluble fraction (Fig. 6, Lane 5,6). Therefore, the insoluble enzyme fraction was solubilized in 8 M urea in the presence of DTT in order to open undesired disulfide bonds. Subsequently, refolding of (R)-HNL was initiated by rapid dilution in renaturation buffer. Gerschitz et al. [55] reported that horse liver ADH could be renatured with 70% yield. For refolding of ADH into the correct structure Zn^{++}-ions are important [56,57]. Initial attempts to refold denatured (R)-HNL purified from the pellet fraction failed. Variation of the Zn^{++} concentration and addition of a glutathione shuffle system [56] were also not successful.

3.4. Sequence similarity of (R)-HNL to alcohol dehydrogenases

A data bank search shows that the (R)-HNL exhibits significant homologies to Zn^{++}-dependent alcohol dehydrogenases (ADH) as already noticed by Trummler and Wajant [50]. A homology search using the program WU-blastp [58] gave highest scores for medium-chain ADHs of class I and III (Table 4, Fig. 7). Class

Fig. 7. Sequence alignment of (R)-HNL from *Linum* with various ADH-sequences of class I and III (see Table 4). The sequence of horse liver ADH I was added for structural comparison. Structurally important glycines are marked with #, ligands of the catalytically important Zn^{++} are marked with ♦; ligands of the structurally important Zn^{++} are marked with ♣ (compare Table 5a). Other catalytically important residues are marked in grey (compare Table 5b and see text). An ADP-binding $\beta\alpha\beta$-motif as described by Ref. [42] is underlined.

III ADHs, which are identical with glutathione-dependent formaldehyde dehydrogenases (FADH) (E.C.1.2.1.1.), are unique among the ADHs in terms of their capacity to oxidize

```
                    1                                                50
R-HNL Linum         MASLPVSFAK PDKNGVITCK AIMLKEAKLP GMSYADTVQI IDIQVDPPQN
ADH I potato        MDSSNPK---------VITCK AAVVGKEG--------EMIKI EEIQVDPPKS
FADH C. malt.       MSESTVGK--------PITCK AAVAWEAAKP--------LSI EDVTVAPPKR
FADH Synch. sp.                           MKSRAAV-AFEVGKPLQI VEIDVAPPQQ
ADH I h. liver                 STAGKVIKCK AAVLWEEKKP--------FSI EEVEVAPPKA

                    51         ♦  ♦                 #♦♦  #      #    100
R-HNL Linum         VELRVKMLCA SVCRTDILTI EGFMAPTQFP KINGHEGVGI IESMGPDTKN
ADH I potato        NEVRIKMLFA SLCHTDIL-A SNGYPYSLFP RVLGHEGVGM IESVGENVTN
FADH C. malt.       HEVRIKLYDT GVCHTDAYTL SGVDPEGAFP VILGHEGAGI VESIGEGVTN
FADH Synch. sp.     GEVLVKITHT GVCHTDAFTL SGDDPEGLFP VVLGHEGAGI VVEVGEGVTS
ADH I h. liver      HEVRIKMVAT GICRSDDHVV SGTLVT PLP VIAGHEAAGI VESIGEGVTT

                    101 #          ♣  ♣  ♣      ♣                    151
R-HNL Linum         FKVGDVIVAP TLGECQTCSS CRSGRTNFCQ NYGANESALE PDGTSRFSYI
ADH I potato        LKEGDIVMPL YLGECKECPN CKSGKSNLCH KYHLTFSGLM LDDTSRISIH
FADH C. malt.       VKVGDHVIAL YTPECGECKF CKSGKTNLCG KIRATQGKGV MPDGTSRFTC
FADH Synch. sp.     VQLGDHVIPL YTAECGKCLF CRSGKTNLCV AVRATQGKGV MPDGTSRFSY
ADH I h. liver      VRPGDKVIPL FTPQCGKCRV CKHPEGNFCL KNDLSMPRGT MQDGTSRFTC

                    151                                            ♦ 200
R-HNL Linum         DSDGKKKLLY YKLGCSTWTQ YMVVDSNYAT KLNEIAPELP PPHGSILSCA
ADH I potato        ----GGQVLY HSFSCSTWSE YIVINANYVI KVDPQK--IP LQHASLLCCG
FADH C. malt.       ----KGKEIL HFMGCSTFSQ YTVVADISVV AINPKAEF-- -DKACLLGCGI
FADH Synch. sp.     ----NGQSLY HYMGCSTFSE YTVVAEVSLA KINPEANH-- -EHVCLLGCGV
ADH I h. liver      ----RGKPIH HFLGTSTFSQ YTVVDEISVA KIDAASP--- LEKVCLIGCGF

                    201            #           #  #                  250
R-HNL Linum         FATGYGAVWL DAAVQEGDSV AIFGVGSVGI SAVIAAKELK AKQIIVVDRN
ADH I potato        FTTGYGATWR EVHVEKGSTV AVLGLGVVGL GAIEGARSQG ASKIIGVDIN
FADH C. malt.       TTGYGAATIT AN VQKGDNV AVFGGGIVGL SVIQGCAERG AAQIILVDIS
FADH Synch. sp.     TTGIGAVHNT AK VQPGDSV AVFGLGGIGL AVVQGARQAK AGRIIAIDTN
ADH I h. liver      STGYGSAVKV AK VTQGSTC AVFGLGGVGL SVIMGCKAAG AARIIGVDIN

                    251         #                                    300
R-HNL Linum         EYKLKMAMEL GATHCINSEK LPEGVTPSQA VRKLTPKEVG VDASIESSGY
ADH I potato        ESKQGKGELF GMTDFINPKE SMTSVSEMIK DVTEG---LG VDYVFECTGI
FADH C. malt.       DKKEEWGQKL GATAFVNPTK LPEGTTIVDK LIEMT--DGG CDFTFDCTGN
FADH Synch. sp.     PAKFELAKQM GATDCINPKD HDQPIQQVIV --EMT--GWG VDHSFECIGN
ADH I h. liver      KDKFAKAKEV GATECVNPQD YKKPIQEVLT --EMS--NGG VDFSFEVIGR

                    301                                              350
R-HNL Linum         DVFMNEAMKA AIHGKAKTVI TGEGIYENDR IFFDFKDFLF GGNVVGNVTG
ADH I potato        PSMLNEAIEA SKLGIGTIVV IGAGHGLTRE -FNLVPLLCG RTLKGSIYGG
FADH C. malt.       VGVMRNALEA CHKGWGTSVI IGVAAAGKEI STRPFQLVTG RTWKGAAFGG
FADH Synch. sp.     VEVMRSALEC AHRGWGQSVI IGVAGAGQEI STRPFQLVTG RKWMGTAFGG
ADH I h. liver      LDTMVTALSC CQEAYGVSVI VGVPPDSQNL SMNPMLLLSG RTWKGAIFGG

                    351                                              400
R-HNL Linum         RVRIHSDFPG LLRKAQEPVI RAGMDKILGY DAATMKCKYE VDIREGTPAL
ADH I potato        -IRLHSDLPA ILHRCATKEI QLNELITHQI SLTEINQSFE LLKDPHCVKI
FADH C. malt.       -VKGRSQLPG IVNNYLDGKL KVEEFITHRE PLAAINKAFE EMHAGDCIRA
FADH Synch. sp.     -VKGRSQLPG MVEQSMRGEI QLAPFVTHTM ELKDINQAFD LMHDGKSIRS
ADH I h. liver      -FKSKDSVPK LVADFMAKKF ALDPLITHVL PFEKINEGFD LLRSGESIRT

                    401                   422
R-HNL Linum         LKALEEVENV DCVKLVIKLN DY
ADH I potato        IIKF
FADH C. malt.       VVDLS
FADH Synch. sp.     VIHY
ADH I h. liver      ILTF
```

S-hydroxymethylglutathione [59] and this substrate interacts with residues additional to, or different from those leading to binding of aliphatic alcohols [60]. Evolutionary studies on ADHs suggest that ADHs of class III are the anchestral form, whereas class I enzymes, the classical mammalian liver enzymes, appears to have arisen by gene duplication during early vertebrate evolution [61,62]. As is demonstrated in Table 5, the conserved amino acid residues found in (*R*)-HNL relative to ADHs include the minimal requirements of the ADH-family [62,63]. These include several structurally important glycines as well as ligands of both the catalytically and structurally important Zn^{++}-ions (Table 5a, Fig. 7), an Asp (Asp 248 in (*R*)-HNL) that determines the specificity for NAD, and a Ser or Thr (Thr 65 in (*R*)-HNL) that facilitates proton removal from the alcohol substrate. By contrast, residues which have been found to be relevant for the substrate specificity of ADHs are not conserved in (*R*)-HNL (Table 5b, Fig. 7). The relation of (*R*)-HNL to both the ancestral class III and the younger class I enzymes further supports the theory that HNL-activity has evolved by divergent evolution from

Table 5
(a) Comparison of catalytically and structurally important amino acid residues identified in alcohol dehydrogenases of class I and III ([62]) with (*R*)-HNL from *L. usitatissimum*: the numbering of residues refers to β-ADH from *Papio hamadrysa* (baboon) [62]

Residue in ADH	Residue in HNL
Structurally important residues in the substrate binding domain, strictly conserved among ADHs	
Gly 66	Gly 84
Gly 71	Gly 89
Gly 77	Gly 95
Gly 86	Gly 104
Val 80	Thr 98
Structurally important residues in the coenzyme binding domain, strictly conserved among ADHs	
Gly 192	Gly 217
Gly 201	Gly 226
Gly 204	Gly 229
Gly 236	Gly 261
Ligands of the catalytically important Zn^{++}	
Cys 46	Cys 63
Asp 49	Asp 66
His 67	His 85
Glu 68	Glu 86
Cys 174	Cys 199
Ligands of the structurally important Zn^{++}	
Cys 97	Cys 115
Cys 100	Cys 118
Cys 103	Cys 121
Cys 111	Cys 129

(b) Residues differentiating between class I and class III ADH-activity compared to (*R*)-HNL

Residues ADH	Residues (*R*)-HNL	Function	References
Ser/Thr 48	Thr 65	essential for ADHI activity and binding of NAD(H), not essential for ADHIII activity	[60,65]
Tyr 93 (III) Phe 93 (I)	Thr 111	amino acid residue responsible for hydrophobicity in the substrate binding pocket	[60,62]
Arg 115 (III) Asp 115 (I)	Gly 133	Arg 115 is relevant for the binding of formyl-glutathione	[66]
Asp 223 (I/III)	Asp 248	coenzyme specificity	[62]

different precursors. Two main evolutionary pathways may be postulated based on the recent data: (*S*)-specific HNLs such as the enzymes isolated from *Sorghum*, *Hevea* and *Manihot*, have evolved from alpha/beta-hydrolases [15,67], whereas (*R*)-specific HNLs, such as the enzymes from *Rosaceae* and *Linum*, have diverged from oxidoreductases [15,50]. The potential structural requirement of (*R*)-HNL for Zn-ions and/or NAD as suggested by the conserved binding motifs requires further investigations. Initial studies have recently been performed by Tummler and Wajant [50]. These authors found no hints for Zn^{++}-binding in the enzyme upon incubation with *o*-phenanthroline at room temperature. Studies of Magonet et al. [64] on the importance of the structural zinc atom for the stability of yeast ADH show, however, that the removal of the catalytically important Zn^{++} requires incubation of yeast ADH in the presence of 100 mM EDTA at 30°C for 2 h. The potential cofactor-requirement of (*R*)-HNL is now under investigation.

Acknowledgements

This work has been supported by the Deutsche Forschungsgemeinschaft (SFB 380). We thank Dagmar Müller for protein sequencing and Simone Münch for helpfull assistance.

References

[1] E.E. Conn, in: P.K. Stumpf, E.E. Conn (Eds.), The biochemistry of plants, Vol. 7, Academic press, New York, 1981, p. 479.
[2] A. Nahrstedt, PI Syst. Evol. 150 (1985) 35.
[3] L. Rosenthaler, Biochem. Zeitschr. 14 (1908) 238.
[4] F. Effenberger, T. Ziegler, S. Förster, Angew. Chem. 99 (1987) 491.
[5] T. Ziegler, B. Hösch, F. Effenberger, Synthesis (1990) p. 575.
[6] F. Effenberger, B. Hösch, S. Förster, T. Ziegler, Tetrahedron Lett. 31 (1990) 1249.
[7] F. Effenberger, B. Hörsch, F. Weingart, T. Ziegler, S. Kühner, Tetrahedron Lett. 32 (1991) 2605.
[8] N. Klempier, U. Pichler, H. Griengl, Tetrahedron: asymmetry 6 (1995) 845.
[9] N. Klempier, H. Griengl, M. Hayn, Tetrahedron Lett. 34 (1993) 4769.
[10] S. Förster, J. Roos, F. Effenberger, H. Wajant, A. Sprauer, Angew. Chem. 108 (1996) 493.
[11] U. Niedermeier, M.-R. Kula, Angew. Chem. 102 (1990) 423.
[12] T. Matsuo, T. Nishioka, M. Hirano, Y. Szuki, K. Tsushima, N. Itaya, H. Yoshioka, Pestic. Sci. 163 (1980) 202.
[13] F. Effenberger, Angew. Chem. 106 (1994) 1609.
[14] H. Wajant, F. Effenberger, Biol. Chem. Hoppe-Seyler 377 (1996) 611.
[15] A. Hickel, M. Hasslacher, H. Griengl, Physiol. Plant. 98 (1996) 891.
[16] J. Vernau, M.-R. Kula, Biotechnol. Appl. Biochem. 12 (1990) 397.
[17] D. Selmar, R. Lieberei, B. Biehl, Plant Physiol. 86 (1988) 711.
[18] E. Swain, J.E. Poulton, Plant Physiol. 106 (1994) 437.
[19] L. Zheng, J.E. Poulton, Plant Physiol. 109 (1995) 31.
[20] H. Wajant, D. Riedel, S. Benz, K.W. Mundry, Plant Sci. 103 (1994) 145.
[21] M. Hasslacher, M. Schall, M. Hayn, H. Griengl, S.D. Kohlwein, H. Schwab, J. Biol. Chem. 271 (1996) 5884.
[22] J. Hughes, F.J.P. De Carvalho, M.A. Hughes, Arch. Biochem. Biophys. 311 (1994) 496.
[23] M. Hasslacher, C. Kratky, H. Griengl, H. Schwab, S.D. Kohlwein, Proteins 27 (1997) 438.
[24] J. Albrecht, I. Jansen, M.-R. Kula, Biotechnol. Appl. Biochem. 17 (1993) 191.
[25] L.L. Xu, B.K. Singh, E.E. Singh, Conn Arch. Biochem. Biophys. 263 (1988) 256.
[26] U. Niedermeier, report no. 2343 Forschungszentrum Jülich, Germany (1990).
[27] U. Kragl, U. Niedermeyer, M.-R. Kula, C. Wandrey, in: H. Okada, A. Tanaka, W.M. Blanch (Eds.), Enzyme Engineering, Vol. X, Ann. NY Acad. Sci., 613 (1990) p. 167.
[28] D.P. Goldberg, in: T.E. Creighton (Ed.), Protein Structure 2nd edn. Information Press, Oxford, 1990, Chap. 10, p. 225.
[29] C.R. Merril, Meth. Enzymol. 182 (1990) 477.
[30] U.K. Laemmli, Nature 277 (1970) 680.
[31] T. Rabilloud, Electrophoresis 13 (1992) 429.
[32] J.L. Lambert, J. Ramasamy, J.V. Paukstelis, Anal. Chem. 47 (1975) 916.
[33] J. Kyhse-Andersen, J. Biochem. Biophys. Meth. 10 (1984) 203.
[34] R. Rudolph, in: H. Tschesche (Ed.), Modern methods in protein and nucleic acid research; Review Articles, Walter de Gruyter, Berlin, Chap. 8, 1990, p. 149.
[35] C. Aaij, P. Borst, Biochem. Biophys. Acta 269 (1972) 192.
[36] R.B. Helling, H.M. Goodman, H.W. Boyer, J. Virol. 14 (1974) 1235.
[37] H. Lehrach, D. Diamond, J.M. Wozney, H. Boedtker, Biochemistry 16 (1977) 4743.
[38] H.C. Birnboim, J. Doly, Nucl. Acids Res. 7 (1979) 1513.
[39] E.M. Southern, J. Mol. Biol. 98 (1975) 503.
[40] M.A. Frohman, M.K. Dush, G.R. Martin, Proc. Natl. Acad. Sci. USA 85 (1988) 8998.
[41] J. Delort, J.B. Dumas, M.C. Darmon, J. Mallet, Nucl. Acid Res. 17 (1989) 6439.

[42] G. Goetz, Master Thesis, University of Cologne, 1995.
[43] C. Bordier, J. Biol. Chem. 25 (1981) 1604.
[44] M. Kehren, Textilindustrie 59 (1957) 13.
[45] A. Sanchez-Ferrer, M. Perez-Gilabert, E. Nunez, R. Bru, F. Garcia-Carmona, J. Chromatogr. A 668 (1994) 75.
[46] A. Sanchez-Ferrer, R. Bru, F. Garcia, Crit. Rev. Microbiol. 29 (1994) 275.
[47] I. Jansen, R. Woker, M.-R. Kula, Biotechnol. Appl. Biochem. 15 (1992) 90.
[48] R. Woker, B. Champluvier, M.-R. Kula, J. Chromatogr. Biomed. Appl. 584 (1992) 85.
[49] J.B. Dumas, Milne Edwards, P. Ravassard, C. Icard-Lipkalns, J. Mallet, in: M.J. McPherson, B.D. Hames, G.D. Taylor (Eds.), PCR 2. A practical appraoch, IRL Press, Oxford, NY, 1995.
[50] K. Trummler, H. Wajant, J. Biol. Chem. 272 (1997) 4770.
[51] J. Albrecht, Master Thesis, University of Düsseldorf, 1991.
[52] H. Eklund, B. Nordström, E. Zeppezauer, G. Söderlund, I. Ohlson, T. Boiwe, B.O. Soderberg, O. Tapia, C.-I. Brändén, J. Mol. Biol. 102 (1976) 27.
[53] T.D. Hurley, W.F. Bosron, J.A. Hamilton, L.M. Amzel, Proc. Natl. Acad. Sci. USA 88 (1991) 8149.
[54] B.J. Takasc, M.-F. Girard, J. Immunol. Meth. 143 (1991) 231.
[55] J. Gerschitz, R. Rudolph, R. Jaenicke, Eur. J. Biochem. 87 (1978) 591.
[56] R. Rudolph, J. Gerschitz, R. Jaenicke, Eur. J. Biochem. 87 (1978) 601.
[57] T. Zhang, H.-M. Zhou, Int. J. Biol. Macromol. 19 (1996) 113.
[58] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, J. Mol. Biol. 215 (1990) 403.
[59] M. Koivusalo, L. Uotila, Adv. Exp. Med. Biol. 284 (1991) 241.
[60] M. Estonius, J.-O. Höög, O. Danielsson, Hans Jörnvall, Biochemistry 33 (1994) 15080.
[61] T. Luque, S. Atrian, O. Danielsson, H. Jörnvall, R. Gonzàlez-Duarte, Eur. J. Biochem. 225 (1994) 985.
[62] H.-W. Sun, B.V. Plapp, J. Mol. Evol. 34 (1992) 522.
[63] R.K. Wierenga, P. Terpstra, W.G.J. Hol, J. Mol. Biol. 187 (1986) 101.
[64] E. Magonet, P. Hayen, D. Delforge, E. Delaive, J. Remacle, Biochem. J. 287 (1992) 361.
[65] H. Eklund, P. Müller-Wille, E. Horjales, O. Futer, B. Holmquist, B.L. Vallee, J.-O. Höög, R. Kaiser, H. Jörnvall, Eur. J. Biochem. 193 (1990) 303.
[66] K. Engeland, J.-O. Höög, B. Holmquist, M. Estonius, H. Jörnvall, B.L. Valle, Proc. Natl. Acad. Sci. USA 90 (1993) 2491.
[67] H. Wajant, K.W. Mundry, K. Pfitzenmaier, Plant Molec. Biol. 26 (1994) 735–746.