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Biocatalytic conversion of unnatural substrates by recombinant almond *R*-HNL isoenzyme 5

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Abstract

Hydroxynitrile lyases (HNLs, EC 4.1.2.10, EC 4.1.2.11, EC 4.1.2.37, EC 4.1.2.39) enantioselectively catalyse the reversible addition of HCN to ketones or aldehydes, thereby forming chiral cyanohydrins, which is of special interest for industrial bio-conversions. We cloned the gene for the HNL isoenzyme 5 (*Pa*HNL5) of the almond tree (*Prunus amygdalus*) and overexpressed it in the methylotrophic yeast *Pichia pastoris*. This opened new ways for the synthesis of (*R*)-cyanohydrins. The characterisation of *Pa*HNL5 revealed high activity for the natural substrate and high enantioselectivity. For further improvement of enzyme properties such as higher activity for the conversion of unnatural substrates, a high throughput cultivation and screening system has been created, which allows the employment of *P. pastoris* as production host for high throughput cultivation and screening of thousands of enzyme variants. The synthesis and cleavage of 2-chlorobenzaldehyde cyanohydrin were used for the demonstration of enzyme activity of recombinant *Pa*HNL5 with a non-natural substrate and for the development of a high throughput screening procedure.

Keywords: Hydroxynitrile lyase; High throughput screening; *Pichia*; Unnatural substrate; Mandelonitrile

1. Introduction

Cyanohydrins are produced by the addition of hydrocyanide to aldehydes or ketones (see Fig. 1) and can be used for the synthesis of many interesting substances such as alpha hydroxy acids, alpha hydroxy ketones and beta amino alcohols, which are important intermediates for the production of pharmaceuticals, vitamins or agrochemicals [1,2]. The production of (*S*)-cyanohydrins on industrial scale was put into practice by the discovery and recombinant production of the (*S*)-hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL) [3,4]. However, its counterpart from the "(*R*)-world", the (*R*)-hydroxynitrile lyase (*Pa*HNL) from almonds (*Prunus amygdalus*) which also shows a broad substrate specificity, did not find its way to large scale applications to date. In the plant family of the Prunoideae, the (*R*)-HNL and their natural substrate (*R*)-(+)-mandelonitrile are separated by compartmentalisation on tissue or subcellular level [5]. The release of HCN from damaged plant tissues is believed to serve as a defense strategy against herbivoral attack [6] or as a nitrogen source for the biosynthesis of L-asparagine [7]. In synthetic chemistry the (*R*)-HNL is applied for the C–C coupling reaction in organic solvents [8], biphasic or emulsion systems [9]. Although the availability or *Pa*HNL in large quantities, mainly from Rosaceae, was described in many publications, e.g. [2,10], the reality reflects a different situation. The catalytic activity of the enzyme is known since 1909 [11] and in fact it was the first enzyme ever described as a catalyst for asymmetric synthesis. Many enantioselective reactions on laboratory scale have been described [10,12,13] in the meantime and there was also interesting progress in the field of reaction techniques [2,12,14,15], extraction from plant sources [16] and also in the field of structural, mechanistical and biochemical

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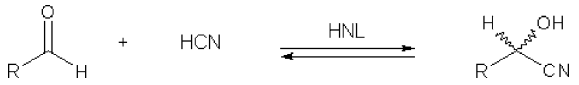

Fig. 1. Reversible addition of HCN to aldehydes and ketones by HNLs.

characterisation of the enzyme [17–19]. Enzyme preparations from plant sources are expensive. The report [16] on cost-effective extraction of (*R*)-HNL from natural sources demonstrates a reasonable progress in the area of native enzyme supply from plant materials, but high amounts of enzyme have to be applied for the desired conversions. Moreover, the application of enzyme extracts from natural sources in multi-ton-scale industrial processes might be prevented by limited supply as well as by consistently changing isoenzyme compositions and protein contents, respectively. Although *Pa*HNL is characterised by broad substrate acceptance, the yield and enantiomeric purity with some aldehydes such as *ortho*-substituted benzaldehyde and acrolein, which are not natural substrates for *Pa*HNL, are low [2,14]. In this paper, we describe the cloning and recombinant expression of a *Pa*HNL isoenzyme, which will serve as a platform for protein engineering strategies towards improved conversion of unnatural substrates. As an example for the reduced activity on slow reacting unnatural substrates, the enzymatic activity for the synthesis of (*R*)-2-chloromandelonitrile in comparison to the unsubstituted mandelonitrile is shown. A kinetic high throughput screening assay for further enzyme development was established.

2. Experimental

2.1. Isolation of genomic DNA from P. amygdalus seeds

Dried almond seeds (Farmgold, lot number L4532, harvest 1999) have been chopped into small fragments, frozen with liquid nitrogen in a peel and accurately powdered in liquid nitrogen, using a pestle. “Breaking buffer” (100 mM NaAc; 50 mM EDTA; 500 mM NaCl, adjusted to pH 5.5; 1.4% SDS and 20 μg/ml RNAse A; 65 °C) was added to 0.1 g frozen powder and incubated for 15 min under constant shaking; through centrifugation (10 min at 7000 × *g*) insoluble cell debris were separated, 10 M ammonium acetate was admixed in the same volume to the supernatant and incubated on ice for 10 min. After centrifugation for 15 min at 10,000×*g* the supernatant has been extracted at first with 2 vol. of phenol/chloroform (1/1, phenol equilibrated with 50 mM Tris, pH 8.0), followed by an extraction with 2 vol. of chloroform/isoamyl alcohol (24/1). Finally the DNA was precipitated from the supernatant with an equal volume of isopropanol. After centrifugation, the DNA-pellet has been washed with 70% ethanol and air-dried. The DNA has been solubilised at 68 °C in 200 μl pure water for 20 min, cleaned via ethanol precipitation (according to Ausubel et al., 1999) and centrifuged. The DNA-pellet was air-dried and solubilised in 50 μl pure water.

2.2. Cloning of hnl genes from P. amygdalus

Amplification and cloning of the *hnl*5 gene: 50 ng genomic DNA as template, 200 ng of each primer pdmdl1st1 (5′-CGGAATTCACAATATGGAGAAATCAACAATGTCAG-3′) and primer pdmdl1e1 (5′-CGGAATTCTTCACATGGACTCTTGAATATTATG-3′), 5 μl of dNTP-mix (each 2 mM), 1.2 U “Hotstar” *Taq* DNA polymerase were mixed with 1× PCR-buffer in a total volume of 50 μl, according to the manual of the “Hotstar Kit” (Quiagen, Hilden, Germany). The following program was used for amplification: 15 min denaturation at 95 °C, followed by 30 cycles with 1 min at 95 °C, 30 s at 64 °C, 1 min at 72 °C, and a final step of 5 min at 72 °C. The DNA-fragment of 2.16 kb in size (analysed via agarose gel electrophoresis) has been purified (“Qiaquick Kit”, Quiagen), directly sequenced using the “Dye Deoxy Terminator Cycling Sequencing” Kit (Applied Biosystems Inc., Foster City, USA), and cloned into pBSSK(−) (Stratagene Cloning Systems, La Jolla, USA) via restriction with *Eco*RI, yielding the plasmid pBSPamHNL5g. The DNA sequence of the genomic PCR product was deposited in the genbank database under the accession number AY321296. Through an overlap extension strategy the introns have been spliced out. In the first round, exons II and III have been amplified with the primers PamHNL5b (5′-GTTCACTCGCTTGCCAATACTTCTGCTCATGATTTTAGCTACTTGAAGTTTGTGTACAACGCCACTG-3′)/PamHNL5c (5′-GATGTATTGGAAGAGAAGAGGATCTTCTCTACT-3′) (PCR 1–1) and PamHNL5d (5′-GATCCTCTTCTCTTCCAATACATCAAATTTGTCAGCTATTGGAGTCATATATACGG-3′)/PamHNL5e (5′-CAACCGGATTGACCTTTCTTGCAGGATTTGAAGGCCCACATACCTTCCTAACATCAGATAGAAGCC-3′) (PCR 1–2), in a total volume of 50 μl including 1× PCR-buffer with 100 pM of the corresponding primers, 2.5 U “Hotstar” *Taq* DNA polymerase (Qiagen), 5 μl of dNTP-mix (each 2 mM), 10 ng of the plasmid pBSPamHNL5g as template, following the program: 15 min at 95 °C, 30 cycles with 1 min at 95 °C, 30 s at 68 °C, 1 min at 72 °C, and a final incubation step of 5 min at 72 °C. The PCR products have been analysed via agarose gel electrophoresis and purified (Qiagen) from the gel. In the second round (PCR 2), roughly 50 ng of the product of PCR 1–1 were prolonged with 100 pM of PamHNL5a2 (5′-CTATTTGTGTTGCATCTTCTTGTTCTTCATCTTCAGTATTCAGAGGTTCACTCGCTTGCCAATACTTC-3′) and PamHNL5c, using the same conditions as mentioned above. This product was purified as well and used as template, combined with the product of PCR 1–2 as second template (each 100 ng), 1× PCR-buffer, 5 μl of dNTP-mix (2 mM each) and 2.5 U “Hotstar” *Taq* polymerase, for a third round without primers (PCR 3), where the two fragments fitted together through their overlapping regions and were amplified with the following pro-

gram: 5 cycles 1 min 94 °C, 30 s 68 °C and 1.5 min 72 °C. For complete extension of the sequence, a fourth PCR (PCR 4) with 100 pM of PamHNL5a1 (5′-GAAGATCTGAATTC-CATGGAGAAATCAACAATGTCAGTTATACTATTTGT-GTTGCATCTTCTTG-3′) and PamHNL5f (5′-AAGATCT-GGAATTCTTCACATGGACTCTTGAATATTATGAATA-GCCTCCAACCGGATTGACCTTTCTTGCAG-3′), both directly added to the reaction mix of PCR 3, was applied, with 20 cycles of 1 min at 95 °C, 30 s at 63 °C, 1.5 min at 72 °C and finally 5 min at 72 °C; conditions were the same as in PCR 1. The products of PCR 4 have been separated on a preparative agarose gel and DNA-fragments in the range of 1.6–1.8 kb were eluted from the gel (Qiagen) and cloned into pBSSK(−) via restriction with *Eco*RI. Several clones were chosen for sequencing and one showed the correct sequence (pBSPamHNL5orf). It served as template (10 ng) for a PCR reaction with 400 ng of PCRHNL5-a (5′-TCGAATTCGA-GCTCGGTACCCGGGGATCCTCTAGAAATAATTTTGT-TTAACTTTAAGAAGGAGATATACATATGGAGAAATC-AACAATGTCAGTTATACTATTTGTGTTGCATC-3′) and 200 ng of PCRHNL5-e (5′-CGAATTCGCCCTTTCGCAT-GCTCACATGGACTCTTGAATATTATGAATAGCCTC-3′), 1× PCR-buffer, 5 μl of dNTP-mix (each 2 mM) and 1.2 U "Hotstar" *Taq* polymerase, with following program: 15 min at 95 °C, 30 cycles with 1 min at 95 °C, 30 s at 68 °C, 1.5 min at 72 °C and final 5 min at 72 °C. After restriction with *Eco*RI and ligation into pBSSK(−), the master template pBSPamHNL5ex has been created, which can be used for expression using *E. coli-P. pastoris*-shuttle vectors.

Amplification of the *hnl*1 gene: the same program as for the amplification of *pa_hnl*5 has been used, with the primers mandlp2f (5′-ACTACGAATTCGACCATGGAGAAATCA-AC-3′) and ecpahnl1e (5′-CTGAATTCAGTTTAAAGAAC-CAAGGATGCTGCTGAC-3′). The PCR-product of 2.16 kb was cloned, sequenced and deposited in the gen-bank database under the accession number AF412329 [17].

2.3. Preparation for heterologous expression of hnl5 in Pichia pastoris

The insert of pBSPamHNL5ex was cloned into the *P. pastoris* gene replacement vector pHILD2 (Invitrogen, San Diego, USA). A clone showing the correct orientation of the gene towards the promoter has been restricted with en-donuclease *Not*I and transformed into *P. pastoris* accord-ing to the "*Pichia* Expression Kit Manual" from Invitro-gen (San Diego, USA). More than 100 histidin-prototroph transformants have been examined for HNL-activity in the culture supernatant after cultivation and methanol induction in liquid cultures according to the kit manual (Invitrogen). After 130 h of cultivation the supernatant was examined for HNL-activity (cleavage of mandelonitrile) with the de-scribed photometrical assay. The most active clone, named *P. pastoris* GS115 PamHNL5-a37, was chosen as a product for further analyses.

2.4. Fermentation of PaHNL5 under controlled conditions

Fermentation of *P. pastoris* GS115 pHILD2_PamHNL5-a37 was done in a 42 l MBR lab-fermentor, applying a three-step procedure: at first, exponential and second lin-ear growth for biomass production, and finally an expres-sion phase for the production of recombinant *Pa*HNL5. About 420 ml of 85% ortho-phosphoric acid, 18 g $CaSO_4$, 286 g K_2SO_4, 244 g $MgSO_4 \cdot 7\ H_2O$, 66 g KOH (all of these of p.a. quality), 1 l glycerol (technical quality), and 20 ml antifoamer 10% Acepol 83E (Carl Becker Chemie GmbH, Germany) (all above components calculated for 20 l) were solubilised in 15 l deionized water (conductiv-ity of 5.5–9.1 μS/cm), filled into the reactor and sterilised at 121 °C for 1 h. After cooling to 29 °C, the pH was ad-justed with 25% ammonia (technical quality) to pH 5.0. After that, 200 ml sterile filtered trace-element solution, containing 16 mg biotin, 480 mg $CuSO_4 \cdot 5H_2O$, 640 mg KI, 240 mg $MnSO_4 \cdot H_2O$, 4 mg $Na_2MoO_4 \cdot 2H_2O$, 1.6 mg H_3BO_3, 440 mg CoCl, 1.6 g $ZnSO_4 \cdot 7H_2O$ and 5.2 g $Fe(II)SO_4 \cdot 7H_2O$ (all of them calculated for 20 l and p.a. quality), was sterile filtrated and added to the reactor. The reactor was inoculated with 2 l preculture, which was cul-tivated at 30 °C according to the manual for the "*Pichia* Expression Kit" of Invitrogen (San Diego, USA). For the fermentation, a constant temperature of 29 °C has been chosen, through aeration (between 15 and 40 l of air per minute) and agitation control (250–500 rpm) the oxygen partial pressure has been kept above 30% of the saturation concentration. After 21 h, the biomass grew up to 22 g/l cell dry weight. Starting from this moment, sterile glycerol has been added in constant small portions in intervals of 15 min, all in all 130 g glycerol/h. During this second, lin-ear growth phase of 42 h, a biomass concentration of 70 g/l has been reached. Subsequently, the third step was started through induction of expression with methanol. A methanol concentration of 0.8–1% (w/v) was adjusted. At the begin-ning and after 2 days of induction, respectively, a further addition of the trace-element solution was carried out (as described above). After 110 h of incubation, an enzyme amount of 110 U/ml culture supernatant was measured. Af-ter separation of the cell through centrifugation, an enzyme preparation was obtained, which was directly applicable to biocatalytic conversion experiments.

2.5. Concentration and purification of HNL5

The culture supernatant was cleaned via crossflow-filtration (0.2 μm) and concentrated by ultrafiltration (Vi-vaflow, Vivascience, Hannover, DE) with a cutoff of 30 kDa up to ∼1 mg protein/ml. The protein sample as well as the column (Q-Sepharose Fast Flow, column volume 10 mL) were equilibrated with binding buffer A (20 mM Piperazin-Cl, pH 5.5). Following program was established on an ÄKTApurifier (Amersham Biosciences, UK Limited, Buckinghamshire, UK): after an initial washing step with

0% elution buffer B (20 mM piperazin-Cl + 1 M NaCl, pH 5.5) without fractionation in 1 column volume (hereinafter CV), a first gradient up to 4% buffer B in 0.5 CV, a second gradient from 4 to 48% buffer B in 1 CV, a third gradient to 60% buffer B in 0.5 CV and a final gradient to 100% buffer B in 1 CV have been applied. Samples were fractionated in 2 ml steps, the flow rate was 2 ml/min. Based on the chromatogram, correct fractions were pooled and further concentrated via Vivaspin concentration tubes (Sartorius, Goettingen, DE).

2.6. Cleavage of racemic mandelonitrile (HNL standard assay)

The enzyme activity was determined at 25 °C by recording the kinetic slope at 280 nm photometrically. A substrate concentration of 12 mM in 0.1 M citrate–phosphate buffer pH 5.0 was used. The enzyme was used in a dilution where a linear slope of ~0.1 per min was observed for the first 5 min. A molar extinction coefficient of 1.376 l/mmol/cm for mandelonitrile was used for the calculation of specific enzyme activity (1 U = 1 μmol of cleaved mandelonitrile).

2.7. High throughput microscale cultivation of P. pastoris

Pichia clones have been individually cultivated in 96 deep-well plates (Bel-Art Products, NJ, USA) in 250 μl BM0.5G medium (0.2 M potassium phosphate, pH 6.0; 13.4 g/l Yeast Nitrogen Base; 5 g/l glycerol; 0.8 mg/l biotin) and shaken at 28 °C with 320 rpm. After 60 h, induction has been started with the addition of 250 μl BM2M (0.2 M potassium phosphate, pH 6.0; 13.4 g/l Yeast Nitrogen Base; 10 mL/l methanol; 0.8 mg/l biotin). Further 10, 24 and 48 h later, induction has been kept via addition of 50 μl BM10M (0.2 M potassium phosphate, pH 6.0; 13.4 g/l Yeast Nitrogen Base; 50 ml/l methanol; 0.8 mg/l biotin). Separation of the cell pellet by centrifugation after 130 h of cultivation yielded the supernatant, which was examined for HNL-activity (cleavage of mandelonitrile) with the described photometrical assay.

2.8. Gram-scale synthesis of mandelonitrile and (R)-2-Cl-benzaldehyde cyanohydrin

Fifteen millimoles of substrate were dissolved in 2.1 ml *tert*-butylmethylether (MTBE). About 150 U of *Pa*HNL5 were diluted with 50 mM buffer (K_2HPO_4/citrate pH 3.4) to an end volume of 3.7 ml. Then the solution was set to a pH of 3.4 with citric acid again and mixed with the substrate/MTBE solution in 20 ml glass vials. The reaction mixture was cooled to 10 °C, 1.2 ml HCN were added and the mixture was stirred with a magnetic stirrer at 700 rpm. After given time-points, 250 μl of the samples were taken, mixed with 100 μl MTBE and centrifuged. Fifty microlitres of the organic phase were used for derivatisation with 30 μl acetyl chloride in presence of 34 μl pyridine and 1 ml dichloromethane for 15 min at room temperature. The samples were analysed by GC using a Chiraldex-column.

2.9. Preparative-scale synthesis of (R)-2-Cl-benzaldehyde cyanohydrine: by PaHNL5

Three hundred millimoles of (42 g) 2-chloro-benzaldehyde were dissolved in 42 ml MTBE. 80 ml (34 U/mmol aldehyde) of *Pa*HNL5 were diluted with 10 ml 200 mM buffer (K_2HPO_4/citrate pH 3.8) and mixed with the substrate/MTBE solution. The reaction mixture was cooled to 10 °C, 19.6 ml (501 mmol) HCN were added in the space over 40 min and the mixture was stirred with a magnetic stirrer at 950 rpm. Cyanohydrins were isolated by repeated extractions using MTBE, and the enantiomeric excess of the products was determined via GC on a cyclodextrine-column, after derivatisation of the cyanohydrins with acetyl chloride.

For comparison of the enantiomeric selectivity of *Pa*HNL5 with enzyme extract from almond seeds three reactions with varying enzyme concentrations were performed at pH 4.0, where both enzymes showed comparable stability: 0.25, 0.5 or 1 ml of the preparation were diluted in 50 mM buffer (K_2HPO_4/citrate pH 4). 0.8 g (5.69 mmol) substrate were solubilised in 3 mL MTBE and added to the enzyme solution. Finally 445 μl (11.38 mmol) HCN were admixed and the whole reaction was stirred at room temperature at 900 rpm.

In both cases, the degrees of conversion and enantiomeric excess were determined via GC on a cyclodextrine-column, after derivatisation of the cyanohydrine with acetyl chloride.

3. Results and discussion

3.1. Cloning and heterologous expression of PaHNL5

Based on the sequence homology to *mdl*1 and *mdl*5 [20] from *P. serotina*, we cloned the genes for the isoenzymes 1 [17] and 5 of *P. amygdalus*. PCR-based splicing of the *hnl*5 gene yielded the intron-free sequence, which was cloned into the cloning vector pBSSK(−). The primary amino acid sequences have been translated from the nucleotide sequences, and comparison showed 76.4% identity between the proteins (see Fig. 2). While HNL1 possesses four potential glycosylation sites, HNL5 has 13 corresponding patterns. Both protein sequences contain typical eukaryotic signal leader sequences, making secretion by eukaryotic host systems very likely. Recently, *Pa*HNL1 was crystallised from the almond seed enzyme preparation and its structure was determined. In the course of this work the *Pa*HNL1 gene was cloned and sequenced [17].

The methylotrophic yeast *P. pastoris* was chosen as a host organism for the heterologous expression of HNL-enzymes, due to its capability of introducing post-translational

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               10        20        30        40        50        60
PaHNL1 MEKSTMSAILLVLYIFVLHLQYSEVHSLATTSDHDFSYLSFAYDATDLELEGSYDYVIVG
       :::::::.::.::...:::::::::::::.:: :::::::.:.:.:::   ::::::::.:
PaHNL5 MEKSTMSVILFVLHLLVLHLQYSEVHSLANTSAHDFSYLKFVYNATDTSSEGSYDYIVIG

               70        80        90       100       110       120
PaHNL1 GGTSGCPLAATLSEKYKVLVLERGSLPTAYPNVLTADGFVYNLQQEDDGKTPVERFVSED
       :::::::::::::::::::.:::::. : :::.:::::::.:::::.::::::::::::::
PaHNL5 GGTSGCPLAATLSEKYKVLLLERGTIATEYPNTLTADGFAYNLQQQDDGKTPVERFVSED

              130       140       150       160       170       180
PaHNL1 GIDNVRGRVLGGTSIINAGVYARANTSIYSASGVDWDMDLVNQTYEWVEDTIVYKPNSQS
       :::::::.:.::::.:::::::::::: :.:: .:..::.:::::::::::.:: ::::.::
PaHNL5 GIDNVRARILGGTTIINAGVYARANISFYSQTGIEWDLDLVNKTYEWVEDAIVVKPNNQS

              190       200       210       220       230       240
PaHNL1 WQSVTKTAFLEAGVHPNHGFSLDHEEGTRITGSTFDNKGTRHAADELLNKGNSNNLRVGV
       ::::   .::::::. :..:::::::: :::.:::::::.:::::::::::::. ::: :.:
PaHNL5 WQSVIGEGFLEAGILPDNGFSLDHEAGTRLTGSTFDNNGTRHAADELLNKGDPNNLLVAV

              250       260       270       280       290
PaHNL1 HASVEKIIFS-NAPGLTATGVIYRDSNGTPHQAFVRSKGEVIVSAGTIGTPQLLLLSGVG
       .:::::::.:: :. .:.: :::: ::.:. :::::::.:::::::::::::::::::::::
PaHNL5 QASVEKILFSSNTSNLSAIGVIYTDSDGNSHQAFVRGNGEVIVSAGTIGTPQLLLLSGVG

     300       310       320       330       340       350
PaHNL1 PESYLSSLNIPVVLSHPYVGQFLHDNPRNFINILPPNPIEPTIVTVLGISNDFYQCSFSS
       :::::::::: ::  .:::::::.:::::::::::::::::: .:::::::: .:.:: :.::
PaHNL5 PESYLSSLNITVVQPNPYVGQFVYDNPRNFINILPPNPIEASVVTVLGIRSDYYQVSLSS

     360       370       380       390       400       410
PaHNL1 LPFTTPPFGFFPSASYPLPNSTFAHFASKVAGPLSYGSLTLKSSSNVRVSPNVKFNYYSN
       :::.::::..::.:::::::::::..:.: ::::.::.::.:::.::..::.:::::::
PaHNL5 LPFSTPPFSLFPTTSYPLPNSTFAHIVSQVPGPLSHGSVTLNSSSDVRIAPNIKFNYYSN

     420       430       440       450       460       470
PaHNL1 LTDLSHCVSGMKKIGELLSTDALKPYKVEDLPGVEGFNILGIPLPKDQTDDAAFETFCRE
        :::..:::::::.:.:: : ::.:::..:. :..::: ::.::::.:::::.::::: .
PaHNL5 STDLANCVSGMKKLGDLLRTKALEPYKARDVLGIDGFNYLGVPLPENQTDDASFETFCLD

     480       490       500       510       520       530
PaHNL1 SVASYWHYHGGCLVGKVLDGDFRVTGINALRVVDGSTFPYTPASHPQGFYLMLGRYVGIK
       .:::::::::: ::::::: .::: ::.:::::::::: : ::::::::::::::..
PaHNL5 NVASYWHYHGGSLVGKVLDDSFRVMGIKALRVVDASTFPYEPNSHPQGFYLMLGRYVGLQ

     540
PaHNL1 ILQERSASDLKILDSLKSAASLVL
       :::::::..   : .  .:
PaHNL5 ILQERSIRLEAIHNIQESM
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Fig. 2. Alignment of protein sequences of *Pa*HNL5 and PaHNL1 [17] isoenzymes from *P. amygdalus*; letters in italic face describe the native plant signal sequence, potential glycosylation sequence patterns are underlined.

modifications and to express and secrete heterologous proteins at high levels [21].

Subsequent to transformation of the construct pHILD2_PamHNL5, more than 100 transformants were cultivated and screened for HNL-activity in the culture supernatant and cellular lysates. After shake-flask cultivation, the most superior clone named PamHNL5-a37 showed an activity of 5 U/ml in the culture supernatant. No activity was found in the cellular lysate of *Pichia* cells, which were washed with 1 M phosphate/citrate buffer pH 5.0 before. This clone was also produced in larger scale in a 42 l bioreactor under controlled oxygen- and carbon source-supply. A highly glycosylated protein was secreted to a high extend by *P. pastoris*. An enzyme activity of 110 U/ml culture supernatant has been measured with mandelonitrile as substrate. Because *P. pastoris* secretes very little amounts of homologous protein, the heterologous protein can be rapidly obtained in high purity via ultrafiltration and anion exchange chromatography. Fig. 3 shows the high purity and high level of glycosylation of the recombinant *Pa*HNL5, directly from the concentrated culture supernatant. For comparison an enzyme preparation from almond seeds was applied for deglycosylation and gel analysis. Endoglycosidase H, leaving one *N*-acetyl-glucosamine-residue attached to the asparagine-residue of the protein backbone, and *N*-glycosidase F, which cleaves between the innermost GlcNAc and asparagines, were used to deglycosylate the enzyme samples, under native as well as denaturing conditions. Both enzymes exhibited complete deglycosylation under denaturing conditions, whereas only endoglycosidase

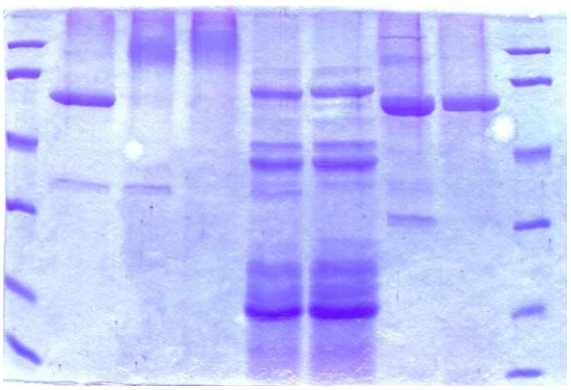

Fig. 3. Standard: molecular weight standard from the *N*-glycosidase F kit from Roche (5 μl = 5 μg); Sample 1: 2 U *Pa*HNL5 with 2.4 U *N*-glycosidase F incubated according to the protocol of the kit; Sample 2: 2 U *Pa*HNL5 with 2.4 U *N*-glycosidase F incubated according to the protocol of the kit but omitting denaturation; Sample 3: 2 U *Pa*HNL5 without deglycosylation; Sample 4: 0.25 U of Roche *R*-HNL preparation grade III from almonds (10.3 U/mg) treated with 2.4 U *N*-glycosidase F under denaturating conditions; Sample 5: 0.25 U of Roche *R*-HNL preparation grade III from almonds (10.3 U/mg) untreated; Sample 6: 2.4 U *Pa*HNL5 incubated with 50 mU endoglycosidase H in 20 mM phosphate buffer, for 12 h at 37 °C without denaturation; Sample 7: 2.4 U *Pa*HNL5 incubated with 50 mU endoglycosidase H in 20 mM phosphate buffer, 0.2% SDS and 0.4% mercaptoethanol for 12 h at 37 °C.

H-treatment allowed deglycosylation under native conditions.

Besides the (*R*)-HNLs from Rosaceae, there are two other (*R*)-HNLs known. The (*R*)-HNL from *Phlebodium aureum* (*Pha*HNL) was described in 1995 [22]. It has no need for cofactors, is a multimer of small 20 kDa subunits and shows a high specific activity with the same natural substrate, namely mandelonitrile, as the Flavin type (*R*)-HNLs from the family of Rosaceae [23,24]. However, only small amounts of enzyme were obtained from this fern [22]. The first cloned (*R*)-*hnl* gene was from *Linum usitatissimun* (*Lu*HNL). This HNL is a Zn^{2+} containing enzyme from the structural class of Zn^{2+} dependent alcohol dehydrogenases and it was expressed in *E. coli* and *P. pastoris* [25,26]. Determined by following the cleavage of acetone cyanohydrine, the production in *E. coli* resulted in 70 U/l fermentation broth (1 U = 1 μmol cleaved substrate per min), what corresponds to the amount of enzyme which can be isolated from 0.8 to 1 kg of plant material [27]. The purified recombinant enzyme showed a specific activity of 76 U/mg in comparison to 53 U/mg which were described for native *Lu*HNL from plant material [28]. Our result with 110,000 U/l of culture supernatant after expression of (*R*)-HNL5 from *P. amygdalus* in *P. pastoris* with mandelonitrile as substrate clearly demonstrates the power of *Pa*HNL which is the preferred (*R*)-HNL for organic synthesis since many years. Even the successful expression of *Lu*HNL in *P. pastoris* did not come close to the results we obtained with recombinant *Pa*HNL5. About 5–20 mg of recombinant *Lu*HNL or 200–800 U (the specific activity was determined to be 40 U/mg using the assay from Selmar et al. [29]) were obtained per liter of culture volume [30]. In addition to its high specific activity, *Pa*HNL showed a broad substrate specificity while most aromatic substrates were not accepted by *Lu*HNL [25]. Low activity and low enantioselectivity of *Lu*HNL was observed for 3-phenyl-propionaldehyde and cinnamaldehyde. Moreover, *Lu*HNL has to be isolated by cell disruption, in contrast to the secreted *Pa*HNL, which can easily be obtained from the culture supernatant in pure form and high concentration.

Table 1
Comparison of recombinant *Pa*HNL5 and almond seed preparation for the synthesis of (*R*)-2-Cl-benzaldehyde cyanohydrin

	*Pa*HNL5		Almond seed-extract		
t (h)	3.5	22	3	3	3
Substrate (U/mmol)	34	34	96	192	384
Yield (%)	71.5	99.7	98	100	100
ee (%)	90.6	90	77.4	81.5	89.1

3.2. Synthesis of (R)-2-Cl-benzaldehyde cyanohydrin

For evaluation of the potential of recombinant *Pa*HNL5 for industrial application, 42 g of 2-chlorobenzaldehyde were converted to 2-chlorobenzaldehyde cyanohydrin. The yield and enantiomeric purity of (*R*)-2-Cl-benzaldehyde cyanohydrin, using different *Pa*HNL preparations, are shown in Table 1. While recombinant *Pa*HNL5 resulted in 71.5% yield with 90.6% ee after 3 h, and 99.7% yield with 90% ee after 22 h, respectively, with an enzyme concentration of 34 U/mmol aldehyde, 10 times more enzyme was necessary from the almond seed enzyme-preparation (EC 4.1.2.10; 2187 U/ml, see Fig. 3, samples 4 and 5) to obtain a similar enantiomeric purity of 89.1% with 100% yield. Three hundred and eighty four units of native enzyme/mmol aldehyde were employed in the reaction mixture. Lower enzyme amounts showed high yields as well, but the enantiomeric purity was low. The recombinant *Pa*HNL5 showed improved technical features. Probably due to higher enzyme stability, high ee was obtained with low amounts of enzyme, although the turnover was slower than with the native enzyme from almond seeds.

For comparison of the conversion of the unnatural substrate (*R*)-2-chlorobenzaldehyde cyanohydrin with the conversion of the natural substrate mandelonitrile, the reactions were performed with small amounts of recombinant *Pa*HNL5. The direct comparison of yields and

Table 2
Comparison of the yield and enantiomeric excess for the synthesis of the natural substrate benzaldehyde cyanohydrin and the technical substrate (*R*)-2-Cl-benzaldehyde cyanohydrin by *Pa*HNL5 after 2 h

Substrate	*Pa*HNL5 (10 U/mmol aldehyde)	
	Yield (%) after 2 h	ee (%) after 2 h
Benzaldehyde	90.3	100
(*R*)-2-Cl-benzaldehyde	15.8	66

enantiomeric purities after the syntheses of mandelonitrile and (*R*)-2-Cl-mandelonitrile is shown in Table 2. The yields have been analyzed after 2 h which means before the substrates have been fully converted. The conversion of the *ortho*-substituted unnatural substrate was significantly slower than with the natural one. The recombinant *Pa*HNL5 showed higher enantioselectivity than the native enzyme isolated from almond seeds. Therefore, the development of a *Pa*HNL5-variant with improved turnover rates for unnatural substrates, in this case (*R*)-2-chlorobenzaldehyde, could be of great interest for commercially viable applications.

3.3. Development of a high throughput cultivation and screening system for P. pastoris

Enzyme development by molecular engineering such as saturation mutagenesis and directed evolution needs the parallel investigation of multiple protein variants. This requires a suitable high throughput cultivation system, which was not available for the *P. pastoris* host. Most attention was paid on reproducibility, reliability and uniform expression levels of the samples. Optimisation of shaking material and conditions, culture media and fermentation times led to a new protocol for the heterologous production of HNL5 in a high throughput manner.

Ninety-six deep-well plates with a plane square bottom served as culture vessels, filled with 500 μl of buffered culture media. The shaking speed, incubation temperature and the cultivation time as well as the induction periods were optimised. The final version, giving the best results, has been used to cultivate *P. pastoris* pHILD_PamHNL5-a37 and induce the production of HNL5. To achieve proper biomass before the induction, cells are grown in glycerol, which at the same time represses the expression of HNL5. For induction methanol was added after the initial media ran out of carbon source and the individual clones reached a uniform cell density. After 130 h of cultivation, the supernatant was harvested through centrifugation and subsequently analysed for activity on the substrate mandelonitrile. The results showed uniform activity throughout the whole plate and were reproducible in further experiments with a standard deviation less than 20%. Comparison with growth and expression behaviour with cultivations in medium scale (shake-flasks) demonstrated good reliability of the deep-well cultivation method. The production of many variants of *Pa*HNL5 in *P. pastoris* in a high throughput system allows the direct comparison of activity performances and forms the basis for a high throughput screening procedure of protein variants for improved turnover with unnatural substrates.

3.4. High throughput activity assay for the cleavage of (R)-2-Cl-benzaldehyde cyanohydrin

Kinetic data over 5 min as well as the absorbance spectra of different wells have been checked, namely after the conversion of the substrate by HNL5-a37 and a negative control, respectively. The biggest difference in absorption has been observed at 300 nm, making this wavelength an attractive target for measuring the ratio of converted and unchanged substrate. Although this substrate is hardly soluble in an aqueous buffer at ambient temperature, heating to ∼55 °C brings it into solution. A 60 mM substrate stock solution of (*R*)-2-Cl-benzaldehyde cyanohydrin was prepared in 50 mM phosphate/citrate buffer pH 3.5 by this way. In order to obtain the final substrate concentration of 12 mM in the assay, we diluted the substrate stock solution into a high salt (1 M) assay buffer at 25 °C before the measurement. By that we obtained an oversaturated substrate solution which allowed a kinetic enzyme activity assay under conditions, where the enzyme was saturated with substrate. A second complication is the rapid substrate decomposition in buffer. This further raises the importance of the developed high throughput screening which allows simultaneous analysis of many variants within short time.

This high throughput screening procedure will allow to screen enzyme variants with improved activity. At the same time, since *P. pastoris* is used as a host for this screening, the selected clones can directly be scaled up for production.

4. Conclusions

The cloning, characterisation and high level production of the isoenzyme HNL5 of *P. amygdalus* opens great application fields for the asymmetric synthesis of cyanohydrins. The enzyme's most interesting distinctive features include high activity on the natural substrate mandelonitrile and high enantioselectivity for the commercially interesting substrate (*R*)-2-Cl-benzaldehyde cyanohydrin. By heterologous expression and secretion using the host *P. pastoris*, almost pure enzyme preparations can be produced by a simple procedure, yielding a predefined protein and thereby omitting difficulties in reproducing conversions with enzyme extracts containing unknown compounds. The development of a high throughput cultivation system for this yeast in combination with a high throughput screening assay for (*R*)-2-Cl-benzaldehyde cyanohydrin provides the basis for further enzyme development in order to overcome limitations of the wild-type enzyme.

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