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Journal of Molecular Catalysis B: Enzymatic 41 (2006) 75–80
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Cloning and optimization of a nitrilase for the synthesis of (3*S*)-3-cyano-5-methyl hexanoic acid

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Received 1 February 2006; received in revised form 24 March 2006; accepted 17 April 2006
Available online 5 June 2006

Abstract

In this paper we describe the cloning and optimization of a nitrilase for a regio- and stereo-specific synthesis of (3*S*)-3-cyano-5-methyl hexanoic acid (**2**) from isobutylsuccinonitrile (IBSN, **1**). Ten representative plant and bacterial nitrilases have been cloned and their substrate specificity was studied using a fluorescent assay. The desired nitrilase AtNit1 from *Arabidopsis thaliana* was identified with high enantioselectivity ($E > 150$). This enzyme was then purified and characterized to be an oligomer of 12 subunits by size exclusion chromatography. AtNit1 was subsequently optimized to increase expression and engineered to improve activity. Preliminary screening of a small percentage (1%) of the mutant library shows that the mutant C236S has a nearly 3-fold increase in reactivity in the hydrolysis of IBSN.

Keywords: Nitrilase cloning; AtNit1; Expression optimization; Fluorescent assay; *Arabidopsis thaliana*; Enantioselectivity; Protein engineering; Oligomerization

1. Introduction

The use of nitrilases to convert nitriles into carboxylic acids has become an increasingly attractive approach to prepare chiral pharmaceutical intermediates. This family of enzymes provide a regioselective and/or enantioselective methodology for nitrile hydrolysis at ambient pressure and temperature, making them ideal as industrial catalysts [1,2]. In this paper we describe the cloning, screening, and engineering of a nitrilase for the synthesis of (3*S*)-3-cyano-5-methyl hexanoic acid (**2**) by a regio- and stereo-specific hydrolysis of racemic isobutylsuccinonitrile (IBSN, **1**) (Scheme 1). The compound **2** can be used as a key intermediate for the preparation of Pregabalin (Lyrica® API), a marketed GABA analog for the treatment of neuropathic pain and partial seizures (Scheme 1) [3–5]. The wrong enantiomer (3*R*)-isobutylsuccinonitrile can be readily epimerized under basic conditions leading to an overall efficient synthetic methodology for the preparation of Pregabalin. In practice, 10 representative plant and bacterial nitrilases were cloned from the gene bank and their substrate specificity and enantioselectivity were studied using a fluorescent assay by capturing the NH_3 byproduct. The desired nitrilase AtNit1 from *Arabidopsis thaliana* was then optimized to increase expression, purified for physical characteirzation and engineered to improve activity.

2. Materials and methods

2.1. Materials

All chemicals of the highest purity were purchased from Aldrich (Milwaukee, WI), Fluka (Buschs, Switzerland) and Sigma (St. Louis, MO). Restriction endonucleases and DNA T4 ligase were obtained from New England Biolabs. Ni-NTA and Superdex 75 column were purchased from Qiagen and Amersham Biosciences, respectively.

2.2. Bacterial strain, plasmids and culture condition

Escherichia coli strains BL21 (DE3), BRLplys (DE3), Rosetta (DE3) and Tuner (DE3) were used as hosts for the recombinant plasmids. pET24 (Novagen) and pET SUMO (Invitrogen) were used for cloning of the recombinant AtNit1. Recombinant *E. coli* strains were cultured at 37 °C overnight in 2 × YT medium containing 16 g/L Bacto tryptone, 10 g/L yeast extract, and 5 g/L NaCl, supplemented with a final concentration

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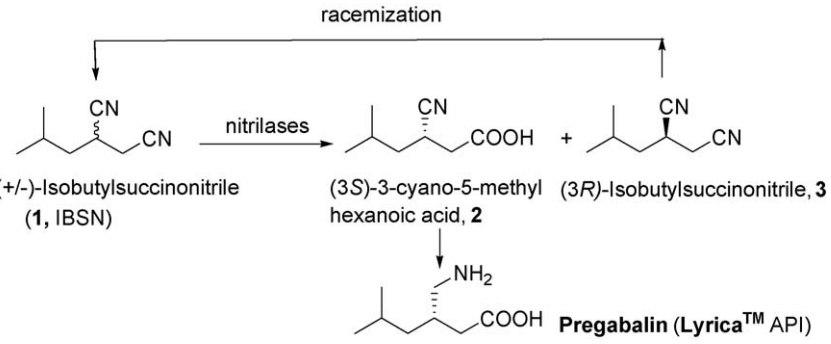
doi:10.1016/j.molcatb.2006.04.010



Scheme 1.

of 50 μg/mL kanamycin. To induce the T7 promoter, isopropyl-thio-β-D-galactoside (IPTG) was added to a final concentration of 0.2 mM when the culture OD is between 0.6 and 0.8.

2.3. Cloning, protein expression and preparation

2.3.1. Cloning of nitrilase genes

Vectors for plasmids construction were purchased from Invitrogen (pET101/D-TOPO and pTrcHis2-TOPO) and Novagen (pET28b(+) and pET36b(+)). Coding regions of AtNit1, AtNit2, and AtNit3 genes were amplified from an *A. thaliana* cDNA library (Stratagene) to generate plasmids pNit03, pNit14, and pNit16, respectively (see Tables 1 and 2 for details). To construct the plasmid pNit06, the AtNit1 gene was amplified from pNit03 DNA. The plasmid pNit15 was constructed by inserting a synthetic codon-optimized (for *E. coli*) AtNit1 gene into pET28b(+) vector (designed, synthesized, and cloned by Blue Heron Biotech). Plasmids pNit07 and pNit08 were synthesized without codon optimization and cloned by Blue Heron. Plasmid pNit09, pNit10, pNit11, and pNit12 were constructed by amplifying corresponding genes from genomic DNA purchased from ATCC. The plasmid pNit13 was constructed by amplifying the corresponding gene from rice cDNA purchased from BioChain.

For the optimization of the expression of AtNit1, its cDNA was cloned into two vectors: pET24 based (Novagen) and pET SUMO (Invitrogen). The expression vector made with pET24 has non-cleavable N-terminal His-tag. The expression vector made with pET SUMO has SUMO fusion protein at its N-terminal for high level expression of soluble AtNit1. To clone AtNit1 gene into pET24, the gene was amplified from pNit15 with the primers nit-p1 (5′-ATTACGGGATCCTCCATATG-AGTACTGTCCAGAACGCTACC-3′) and nit-p2 (5′-CTAA-AGCTTATTTATTAGAATCATCTTCGGC-3′). Using BamH1 site at 5′-end and Xhol site at 3′-end AtNit1 was inserted into the corresponding sites of pET24. To clone AtNit1 gene into the pET SUMO vector, the gene was amplified from

Table 2
Primer sequences for nitrilase cloning

Primer name	Oligonucleotide sequence (5′–3′)
Nit8	CTATTTGTTTGAGTCATCCTCA
Nit9	CACCATGTCTAGTACTAAAGATATGTCAACT
Nit11	CACCATGTCAACTTCAGAAAACACTCCGT
Nit12	CTACTTGTTTGAGTCATCTTCC
Nit13	CACCATGTCTAGTACTGAAGAAATGTCATCA
Nit14	CTATTTGTTTGATTCATCCTCT
Nit17	CATATGTCTAGTACTAAAGATATGTCAACT
Nit20	CATATGGCTATGGTCCCCTCGGGCTC
Nit21	TTAGTAGGGCTTTGCAGTGCTGTCAC
Nit30	GATCCATATGACAACACATCGAATCGCCGT
Nit31	GATCAAGCTTCATCTAGGGTTTGAGCGTGGT
Nit32	GATCCCATGGGGTTCTGGAAAGTTGCAGCAG
Nit33	GATCAAGCTTCTAGCGTAATGGAATGATATCGC
Nit34	GATCCCATGGGTGTCGCACTAGCACAACTTA
Nit35	GATCAAGCTTCCCCAATTTACGCTTCAG
Nit36	GATCCCATGGGTAAGTTGAAAGTCGCGGCAGT
Nit37	GATCAAGCTTGCGCCGCGCTTAGTTC

Table 1
List of plasmids containing nitrilase genes from bacteria and plants

Plasmid name	Insert gene	Protein encoded	Organism	Vector name	Cloning sites	PCR primers
pNit03	AtNit1	NP_851011	*A. thaliana*	pET101/D-TOPO	TOPO	Nit8, Nit9
pNit06	AtNit1	NP_851011	*A. thaliana*	pTrcHis2-TOPO	TOPO	Nit8, Nit17
pNit07	BnNit2	AAK57436	*Brassica napus*	pET28b(+)	NcoI–HindIII	n/a
pNit08	ZmNit1	AAO11743	*Z. mays*	pET28b(+)	NcoI–HindIII	n/a
pNit09	BB1116	NP_887662	*B. bronchiseptica*	pET36b(+)	NdeI–HindIII	Nit30, Nit31
pNit10	yobB	NP_416357	*E. coli* (*k12*)	pET28b(+)	NcoI–HindIII	Nit32, Nit33
pNit11	lp_0433	NP_784228	*Lactobacillus plantarum*	pET28b(+)	NcoI–HindIII	Nit34, Nit35
pNit12	RAP4166	NP_949502	*R. palustris*	pET28b(+)	NcoI–HindIII	Nit36, Nit37
pNit13	OsNit	XP_466925	*O. sativa*	pTrcHis2-TOPO	TOPO	Nit20, Nit21
pNit14	AtNit2	NP_190016	*A. thaliana*	pET101/D-TOPO	TOPO	Nit11, Nit12
pNit15	AtNit1	NP_851011	*A. thaliana*	pET28b(+)	NcoI–HindIII	n/a
pNit16	AtNit3	NP_190018	*A. thaliana*	pET101/D-TOPO	TOPO	Nit13, Nit14

n/a, not applicable.

pNit15 with the primers nit-TA-P1 (5′-ATGAGTACTGTCC-AGAACGCTACC-3′) and nit-p2 (5′-CTAAAGCTTATTTAT-TAGAATCATCTTCGGC-3′). The PCR product was directly ligated into a pET SUMO TA cloning vector. The resulting expression vectors were sequence confirmed and transformed into various *E. coli* strains for expression. To express the recombinant gene for small scale detection, both vectors were transformed into *E. coli* strains (BL21 (DE3), BRLplys (DE3), Rosetta (DE3), Tuner (DE3)) for over expression of AtNit1. Over night cultures were performed in 2 × YT medium at 37 °C, diluted 1:20 to 5 mL fresh 2 × YT containing 50 μg/mL Kanamycin. When OD of cultures is between 0.6 and 0.8, IPTG was added to a final concentration of 200 nM. Cultures were then performed at 23 °C over night for the expression of AtNit1. About 1 mL of each culture before and after induction was saved and pellets were stored at −20 °C for expression analysis. Cell pellets were lysed by sonication in a buffer containing 20 mM Tris–Cl pH 8.0, 300 mM NaCl, 20 mM imidazole, and 14 mM β-mercaptoethanol and recombinant AtNit1 was recovered by 40 μL of Ni-NTA resin (Qiagen). Expressed protein was analyzed by SDS-PAGE. To scale up, 1.5 L culture was induced per flask. The growth and the induction conditions were the same as the small scale.

To purify larger quantity of AtNit1, cell pellets from a 3 L culture of AtNit1 in pET24 were resuspended with lysis buffer containing 25 mM Tris–HCl, pH8.0, 300 mM NaCl, 30 mM imidazole, pH 8.0, 14 mM β-mercaptoethanol. Cells were lysed by microfluidization and clear supernatant was obtained by ultracentrifugation at 40,000 rpm for 45 min at 4 °C. The supernatant was loaded on prewashed 20 mL Ni-NTA column. The column was washed with lysis buffer containing 100 mM imidazole until the absorbance reached the baseline. AtNit1 was eluted with 400 mL linear gradient of 100–400 mM imidazole in lysis buffer. The peak fractions were pooled (56 mL at 0.2 mg/mL) and concentrated to 8 mL and loaded on Superdex 75 sizing column. AtNit1 was eluted overnight at 0.5 mL/min in a phosphate buffer containing 50 mM NaCl phosphate, pH 7.8, 300 mM NaCl, 2 mM EDTA, and 5 mM DTT. The peak fractions were pooled and the buffer was exchanged into a storage buffer containing 25 mM Tris–HCl pH 7.5, 2 mM EDTA, and 5 mM DTT. The final protein concentration was measured with protein reagent (Pierce) using BSA as a standard. The final purified protein was then used for enzyme activity assays, and characterized by light scattering, size exclusion chromatography, and native PAGE.

2.3.2. Determination of native AtNit1 molecular weight

Purified AtNit1 was analyzed by size exclusion chromatography on a Superdex 200 gel filtration column using an AKTA FPLC system calibrated with the protein standards ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and blue dextran 2000 (void volume; ∼2000 kDa). The running buffer was 25 mM HEPES, pH 7.5, containing 150 mM NaCl and 0.25 mM TCEP with a flow rate of 1.5 mL/min. The protein was also analyzed by DLS (Protein Solution's DynaPRO), analytical size exclusion chromatography coupled to a Wyatt DAWN EOS MALS detector, and native PAGE under identical buffer conditions.

2.3.3. Analytical size exclusion chromatography conditions

Purified AtNit1 was separated by size exclusion chromatography on a Superdex 200 gel filtration column using a Dionex HPLC system. The running buffer was 25 mM HEPES, pH 7.5, containing 150 mM NaCl, 0.25 mM TCEP.

2.3.4. Mutant library construction and enzyme preparation for screening

The mutagenesis reaction was carried out using the GeneMorph II Random Mutagenesis kit (Stratagene), following manufacturer's protocols. Primers used for the PCR reaction were pTrcHis Forward (5′-GAGGTATATATTAATGTATCG-3′) and pTrcHis Reverse (5′-GATTTAATCTGTATCAGG-3′). The pNit06 DNA was used as the template. Amplified PCR fragments were digested with NcoI and HindIII and cloned into the pNit06 vector digested with the same enzymes. The constructed library was transformed into Top10 electro-competent cells (Invitrogen). The transformed cells were plated then on a LB agar plate with 100 μg/mL ampicillin and incubate at 37 °C overnight. Individual colonies were picked and inoculated into 96-well plates containing 1 mL of LB and 100 μg/mL ampicillin and sealed with Air Pore Tape (Qiagen). The culture was incubated at 37 °C overnight and shaken at 250 rpm. Next day, 160 μL of the culture was transferred into 0.8 mL LB with 100 μg/mL ampicillin in a 96-well plate, and incubated at 37 °C with shaking for 3 h. About 20 μL of IPTG at a concentration of 10 mM was added to each well and the culture was incubated at 30 °C overnight with shaking. Cells were collected by centrifugation and resuspened in 100 μL of BugBuster with Benzonase (Novagen). The plate was incubated at room temperature and shaken at 650 rpm for 1 h, and the cell lysate was subjected to activity screening.

2.4. Enzyme assay for nitrilase activity

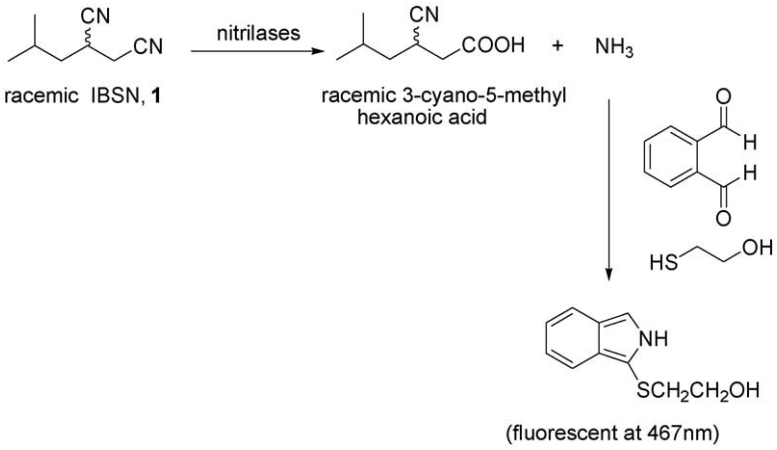
2.4.1. Enzymatic activity assay

The nitrilase activity of the mutant library was determined by a fluorometric assay (Scheme 2) [6]. In brief, 20 μL cell lysate and 10 μL of IBSN (100 mg/mL in DMF) were added to 150 μL of nitrilase reaction buffer (50 mM KPO_4, pH7.5, 2 mM DTT, 1 mM EDTA). The reaction mixture was incubated and shaken at 650 rpm at room temperature for 2 h. About 10 μL of the nitrilase reaction mixture was then transferred to 150 μL of buffered *o*-phthaldialdehyde/2-mercaptoethanol solution. The fluorescent complex was allowed to develop for 30 min at room temperature with shaking at 650 rpm. The fluorescence intensity was measured with excitation and emission wavelengths of 412 and 467 nm, respectively.

3. Results and discussion

3.1. Substrate specificity

Initial screening showed that the crude nitrilase from *A. thaliana* (ordered from Julich Fine Chemicals) showed excellent enantioselectivity (98% ee, 45% conversion) by chiral Gas Chromatography (GC) (Chiraldex™ G-TA, 30 m × 0.25 mm)



Scheme 2.

though with poor activity toward IBSN. Because of these semi-encouraging results, we decided to clone other nitrilases with high sequence homology to AtNit1. From a BLAST search, we selected several proteins with confirmed nitrilase activity as well as some putative sequences suspected to have nitrilase activity because of sequence similarity (Table 1).

Primary screening of 10 nitrilases was carried out by fluorescence assay described above and further analysis by chiral GC. The best one is AtNit1 with the highest activity and excellent enantioselectivity (98% ee, 45% conversion). Unfortunately, the activity of AtNit1 is still too low to be an efficient catalyst for IBSN conversion and thus we decided to optimize the expression and engineer the activity as described in the next section. It should be noted that screening showed that ZmNit1 from *Zea mays* and OsNit from *Oryza sativa* are also active toward IBSN. The fact that Nit09 from *Bordetella bronchiseptica* and Nit12 from *Rhodopseudomonas palustris* are both active toward a variety of other substrates including hydrocinnamonitrile, suggests that they are true nitrilases, but with poor specificity toward the desired IBSN substrate.

3.2. Expression optimization of AtNit1

The nitrilase AtNit1 from *A. thaliana* was previously cloned and its expression in *E. coli* was shown to be undetectable with crude extracts [7]. A synthetic gene with codons optimized for *E. coli* expression was ordered from Blue Heron Biotechnologies. However, no significant improvement in the expression level was observed. In order to obtain increased expression of AtNit1, we cloned AtNit1 into pET24 with N-terminal 6× His-tag and also pET SUMO and transformed them into several *E. coli* strains. Expression of AtNit1 in crude extracts and recovery by Ni-NTA resin was analyzed on SDS-PAGE (Fig. 1, only AtNit1 in pET24 was shown). Induced expression was detectable in whole cell extracts in most strains tested but undetectable in the supernatant indicating AtNit1 was mostly insoluble (Fig. 1A–C; lanes W). However, AtNit1 could be detected from the enriched supernatant following Ni-NTA purification. AtNit1 was then purified from a 3 L culture of transformed Tuner cells (Fig. 2).

AtNit1 was purified from Tuner cell lysate by Ni-NTA affinity chromatography and Superdex 75 sizing column. Clear cell lysate was loaded over prewashed 20 mL of Ni-NTA column and unbound proteins were washed with lysis buffer containing 100 mM imidazole. Tightly bound AtNit1 on Ni-NTA was eluted with a linear gradient of 100–400 mM imidazole in lysis buffer. Pooled protein was then further purified by Superdex 75 size exclusion chromatography. Purified AtNit1 has a mass of 39.2 kDa and under our expression and purification conditions we were able to obtain 6 mg of AtNit1 (>95% purity) from a 3 L cell culture.

With pure AtNit1 in hand, we decided to study its physical properties in more detail. Size exclusion chromatography shows that it is an oligomeric protein of about 12 subunits and the elution volume corresponds to a molecular weight of ∼440 kDa (Fig. 3(a)), which was supported by native PAGE (data not shown) and light-scattering studies (DLS and multi-angle light scattering or MALS). Interestingly, AtNit1 seems to exist in multiple oligomeric forms in solution as the protein eluted as a broad peak during size exclusion and yielded multiple bands on native PAGE (not shown). MALS data confirmed this phenomenon (Fig. 3(b)) and showed that the elution peak from size exclusion was not homomeric and consists of higher molecular weight species at the beginning followed by a tailing off to lower molecular weight species at the end of elution. We have seen similar oligomerization phenomena for other nitrilases from our lab, and experiments are currently underway to determine if oligomerization is necessary for nitrilase activity [8].

3.3. AtNit1 engineering by ePCR

Protein engineering has been proven to be a powerful tool in generating biocatalysts for industrial application. We decided to explore the potential of improving the AtNit1 activity towards IBSN by applying the engineering technology. A mutant library of AtNit1 was generated by random mutagenesis (see Section 2). Analysis of 96 clones by PCR indicated that more than 98% of the clones in the library contain the AtNit1 gene (data not shown). By analysis of the 42.4 kb nucleotide sequence data obtained from the library, a total of 60 nucleotide mutations

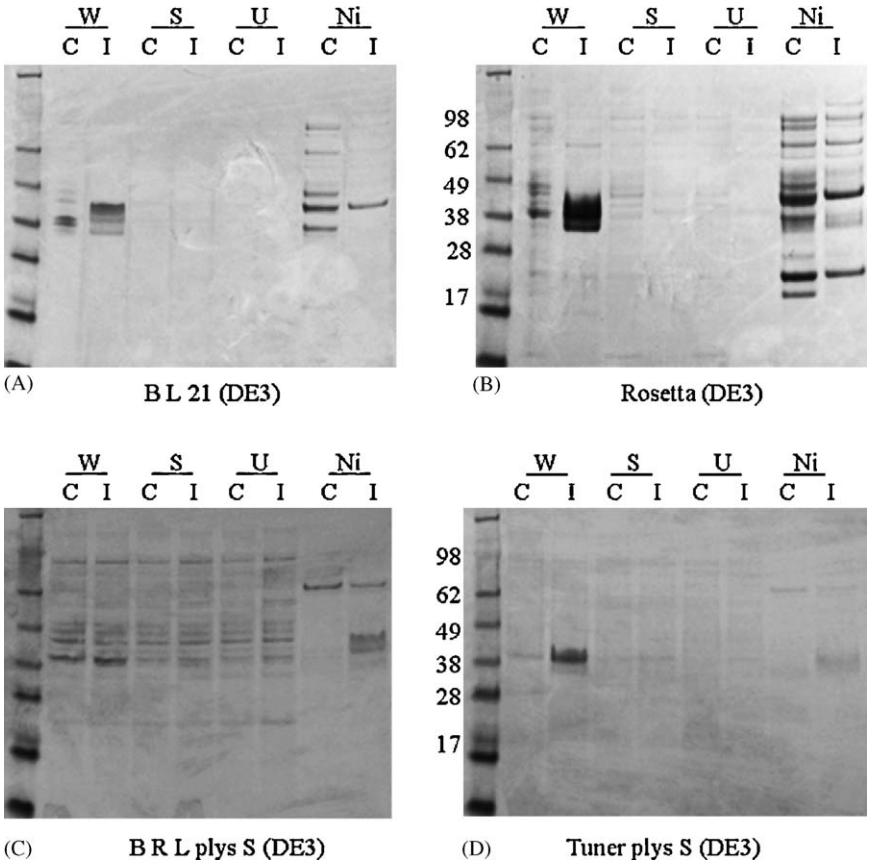


Fig. 1. Analysis of Nit1 expression in various *E. coli* strains: whole cell extract (W), supernatant (S), Ni-unbound (U), and Ni-pull down (Ni) of AtNit1 (in pET24) from BL21 (DE3), BRLplys (DE3), Rosetta (DE3), Tuner (DE3) were analyzed by SDS-PAGE and strained with Coomassie (C for control samples without IPTG induction; I for IPTG induced samples).

were identified. Distribution of mutations is shown in Fig. 4. From this data, it was calculated that the average mutation of the library is 1.4 mutations/gene, and the library contains 10^6 possible variants. An activity screening experiment was carried out on 110 96-well plates containing 9962 mutant clones and 598 controls (wild-type AtNit1 and non-expression clones). Eight clones were identified and confirmed to have at least 130% activity comparing with the wild-type parent. Nucleotide sequence analysis of these clones showed that each clone contains 1–3 amino acid miss-sense mutations (Table 3). Clone P90D8 showed a nearly 3-fold increase of activity compared with the wild-type parent while still retaining the same stereoselectivity. It contains a single miss-sense mutation of C236S. The same C236S mutation also occurs in the clone P70F5. Since the activity is decreased in the clone P70F5 comparing with P90D8, one or both mutations of P172S and V291I may contribute negatively to the enzymatic activity. Since only 1% of the possible library was screened, better variants of the enzyme could probably be obtained by

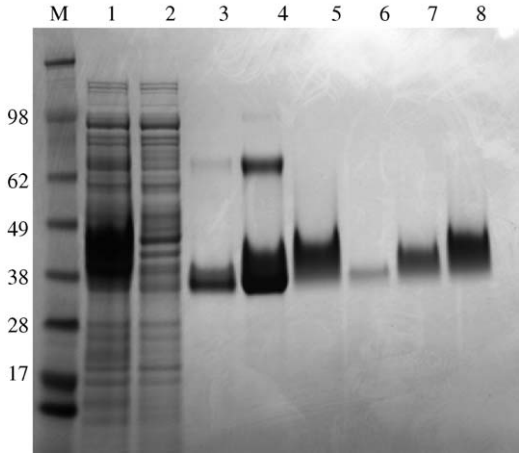


Fig. 2. AtNit1 purification summary by SDS-PAGE (M, molecular weight standard (kDa); lanes 1–8 representing whole cell extracts, supernatant, Ni Pool, sizing 75 load, sizing pool, 1 μg pure protein, 5 μg pure protein, and 10 μg pure protein, respectively).

Table 3
Summary of improved AtNit1 variants

Clone	Relative activity	Nucleotide mutation	Amino acid mutation
WT	100	–	–
P90D8	272	T333A, T706A	A111A, C236S
P48F4	213	A85C	I29L
P67D10	189	C265A, A537T	R89S, G179G
P70F5	184	C514T, G707C, G871A	P172S, C236S, V291I
P102E11	176	C265A, G657A	R89S, S219S
P54C6	147	T303A, T715A, T738A	H101Q, C239S, D246E
P73G12	137	T28S	A200G
P41B10	130	A82T	T28S

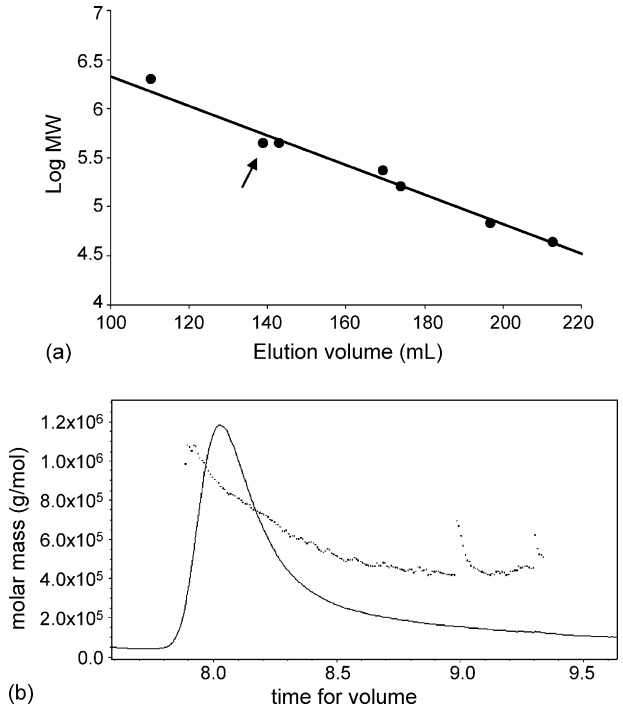


Fig. 3. Native molecular weight analysis of AtNit1. (a) Size exclusion chromatographic analysis of purified AtNit1 (Superdex 200 gel filtration column, running buffer 25 mM HEPES, pH 7.5, containing 150 mM NaCl, 0.25 mM TCEP; 1.5 mL/min flowrate). Estimation of the native molecular weight based on calibration with the mass standards ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and blue dextran 2000 (void volume; ~2000 kDa). The estimated AtNit1 molecular weight in solution is ~440 kDa (arrow on graph), corresponding to 12 subunits. (b) Analytical size exclusion coupled to a MALS detector showing that AtNit1 elutes as a broad peak composed of multiple oligomeric species (solid line, UV at 280 nm; dotted line, molecular weight).

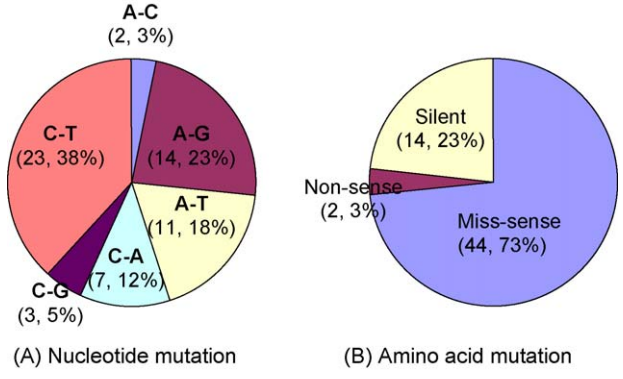


Fig. 4. Distribution of nucleotide and amino acid mutations of the AtNit1 library. Nucleotide mutations (A) and amino acid mutations (B) of the analyzed sequences are shown with the number and percentage distribution indicated in parentheses.

screening more clones from the library. Alternatively, combination or further engineering of existing improved variants may result in better variants.

3.4. Conclusion

Ten nitrilases have been cloned from bacterial and plant species for a regio- and enantioselective hydrolysis of racemic isobutylsuccinonitrile, a useful chiral intermediate for the synthesis of Pregabalin. The best nitrilase is AtNit1 from *A. thaliana* with a high *E*-value (>150). Its expression was optimized and the enzyme was purified by size exclusion chromatography to allow a more detailed characterization as an oligomer of approximate 12 subunits. Furthermore, preliminary work has been carried out to improve the activity of this enzyme by ePCR using a high throughput fluorescent assay. One percent of the mutant library was screened and eight mutants showed improved activity. The C236S mutant was confirmed to have a nearly 3-fold increase in activity as compared to the wild-type parent while retaining the same enantioselectivity. This nitrilase-catalyzed conversion of IBSN has been scaled up to 5–10 g [9], and by reducing the cost of the nitrilase nearly 3-fold, the improvement in reactivity can make the chemoenzymatic process for the production of Pregabalin more cost effective compared to the existing chemical processes [4,5].

Acknowledgments

The authors gratefully acknowledge John Wong, Michael Burns for providing chemical reference standards and experimental data on scaling up, Anita Gujral, Paulo Durao, Shanghui Hu and Carlos Martinez for their fruitful discussions.

References

[1] (a) L. Martinkova, V. Mylerova, Curr. Org. Chem. 7 (2003) 1279–1295; (b) D. Brady, A. Beeton, J. Zeevaart, C. Kgaje, F.V. Rantwijk, R.A. Sheldon, Appl. Microbiol. Biotechnol. 64 (2003) 76–85.
[2] C. O'Reilly, P.D. Turner, J. Appl. Micro. 95 (2003) 1161–1174.
[3] T.Z. Su, M.R. Feng, M.L. Weber, J. Pharm. Exp. Ther. 313 (2005) 1406–1415.
[4] M. Burk, P. De Koning, T. Grote, M. Hoekstra, G. Hoge, R. Jennings, W. Kissel, T. Le, I. Lennon, T. Mulhern, J. Ramsden, R. Wade, J. Org. Chem. 68 (2003) 5731–5734.
[5] M.S. Hoekstra, D.M. Sobieray, M.A. Schwindt, T.A. Mulhern, T.M. Grote, B.K. Huckabee, V.S. Hendrickson, L.C. Franklin, E.J. Granger, G.L. Karrick, Org. Proc. Res. Dev. 1 (1997) 26–38.
[6] A. Banerjee, R. Sharma, U.C. Banerjee, Biotechnol. Appl. Biochem. 37 (2003) 289–293.
[7] S. Steffen, H. Wajant, F. Effenberger, Eur. J. Biochem. 269 (2002) 680–687.
[8] Y. Tong, D. Hughes, L. Placanica, M. Buck, Structure 13 (2005) 7–13.
[9] M. Burns, J. Weaver, J. Wong, WO 2005100580.