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Cloning, overexpression, and characterization of a thermoactive nitrilase from the hyperthermophilic archaeon *Pyrococcus abyssi*

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Abstract

Four open reading frames encoding putative nitrilases were identified in the genomes of the hyperthermophilic archaea *Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Pyrococcus furiosus*, and *Aeropyrum pernix* (growth temperature 90–100 °C). The nitrilase encoding genes were cloned and overexpressed in *Escherichia coli*. Enzymatic activity could only be detected in the case of *Py. abyssi*. This recombinant nitrilase was purified by heat treatment of *E. coli* crude extract followed by anion-exchange chromatography with a yield of 88% and a specific activity of 0.14 U/mg. The recombinant enzyme, which represents the first archaeal nitrilase, is a dimer (29.8 kDa/subunit) with an isoelectric point of p*I* 5.3. The nitrilase is active at a broad temperature (60–90 °C) and neutral pH range (pH 6.0–8.0). The recombinant enzyme is highly thermostable with a half-life of 25 h at 70 °C, 9 h at 80 °C, and 6 h at 90 °C. Thermostability measurements by employing circular dichroism spectroscopy and differential scanning microcalorimetry, at neutral pH, have shown that the enzyme unfolds up to 90 °C reversibly and has a T_m of 112.7 °C. An inhibition of the enzymatic activity was observed in the presence of acetone and metal ions such as Ag^{2+} and Hg^{2+}. The nitrilase hydrolyzes preferentially aliphatic substrates and the best substrate is malononitrile with a K_m value of 3.47 mM.

Keywords: Nitrilase; Archaea; Expression; Purification

Nitrile compounds (R-CN) are widespread in the environment and are mainly present as cyanoglycosides or cyanolipids, which are produced by plants and insects [1]. The microbial degradation of nitriles proceeds through enzymatic processes that involve nitrilases or nitrile hydratases. Nitrilases (EC 3.5.5.1) are thiol enzymes that hydrolyze nitriles directly to the corresponding carboxylic acids with release of ammonia whereas nitrile hydratases (EC 4.2.1.84) catalyze the hydration of nitriles to amides.

The first nitrilase was discovered nearly 40 years ago in plants [2,3]. Nitrilases have been also found in bacteria of the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Nocardia*, *Pseudomonas*, and *Rhodococcus* [4–6], fungus *Fusarium* [7,8], and plants [9–12]. Recently, a nitrilase was described as fusion protein with the nucleotide-binding protein fragile histidine triad in invertebrates [13]. Active nitrilases were also obtained from genomic libraries constructed from the "metagenome" of uncultured microorganisms [14]. To the best of our knowledge there are no reports on the production of nitrilases in archaea.

Nitrilases exhibit different physicochemical characteristics and a diverse substrate spectrum. According to the substrate spectrum, nitrilases can be subdivided into three categories [5,15]: (i) nitrilases that hydrolyze aromatic or

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heterocyclic nitriles (e.g., nitrilase from *Arthrobacter* sp.), (ii) nitrilases that preferentially hydrolyze aliphatic nitriles (e.g., nitrilase from *Rh. rhodochrous* K22 [16]), and (iii) nitrilases that hydrolyze preferentially arylacetonitriles (e.g., arylacetonitrilases from *Ps. fluorescens* [15] and *Alcaligenes faecalis* JM3 [17]).

Nitrilases are generally inducible enzymes and are composed of subunits which vary in size and number [5,6]. It has been observed that in most cases different subunits of nitrilase self-associate to form an active enzyme and this association is accelerated by various factors, including temperature [18], enzyme concentration [18], presence of substrate [19,20], and salt or organic solvent [20].

Nitrilases do not require any metal co-factor or prosthetic group for activity. They are reported to have catalytically essential cysteine residues at, or near, the active site [16,21]. A possible mechanism for the nitrilase-catalyzed reaction indicates a nucleophilic attack by a thiol group on the carbon atom of the nitrile with concomitant protonation of nitrogen to form a tetrahedral thiomidate intermediate. Subsequent steps involve attack by two water molecules and protonation of the nitrogen atom, which is lost as ammonia. In some cases, the tetrahedral intermediate formed can break down anomalously to produce amide instead of the normal acid product [19].

The versatile biocatalytic nature and applications of nitrile-converting enzymes are now increasingly recognized for the production of several pharmaceuticals and fine chemicals. Nitrile-degrading enzymes also play a significant role in the protecting of the environment due to their capability to eliminate highly toxic nitriles. The advances in biosynthetic regulation, genetics, and structure-function relationships of nitrile-metabolizing enzymes will lead to improved properties such as higher enzyme activity, higher tolerance to substrate and products, and higher stability of the biocatalysts used in commercial processes [5,6]. Running such processes at elevated temperatures has also many advantages, including significant improvement of transfer rates, higher substrate solubility, and reduced risk of contamination. Very little, however, is known about nitrilases that are active at high temperatures. Two moderately thermoactive nitrilases described so far were isolated from *Acidovorax facilis* 72W [22] and *Bacillus pallidus* Dac521 [23].

In this paper, we report on the cloning, expression, and purification of a thermoactive nitrilase from the hyperthermophilic anaerobic archaeon *Pyrococcus abyssi* that grows optimally at 100 °C.To our knowledge this is the first archaeal nitrilase described so far.

Materials and methods

Materials

All chemicals were obtained from Sigma–Aldrich (Taufkirchen, Germany) and Merck (Darmstadt, Germany) in the highest analytical grade. All enzymes used for cloning procedures were obtained from New England Biolabs (USA). Oligonucleotides were synthesized by MWG (Munich, Germany). The archaeal strain *Py. abyssi* GE5 (CNCM I-1302) was supplied by CNCM (Collection Nationale de Cultures de Microorganismen, France).

Cell growth

The archaeon *Py. abyssi* was cultivated anaerobically at 100 °C in the following medium (pH 7.0) containing (per liter): 23.4 g NaCl, 10.8 g $MgCl_2{\cdot}6H_20$, 4.0 g Na_2SO_4, 0.7 g KCl, 0.2 g $NaHCO_3$, 0.2 g $CaCl_2{\cdot}2H_2O$, 0.09 g KBr, 0.025 g $SrCl_2{\cdot}6H_2O$, 0.025 g H_3BO_3, 0.003 g NaF, 5 g elementary sulphur, 1 g yeast extract, 4 g peptone, 0.35 g KH_2PO_4, 0.7 g NH_4Cl, and 10 ml trace element solution according to [24].

The *Escherichia coli* cells were grown in Luria–Bertani medium (pH 7.0): 10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g NaCl. The antibiotics were used at the following concentrations: ampicillin at 100 μg/ml and chloramphenicol at 34 μg/ml. The nitrilase expression was induced at a cell density of about 0.8 (OD_{600nm}) by the addition of isopropyl-β-D-thiogalactopyranosid (IPTG[1], 1 mM). After 4 h of induction the cells were separated from the medium by centrifugation in a GLS Sorvall rotor at 3045*g*, at 4 °C for 10 min. The bacterial pellets were washed with a phosphate buffered saline (per liter, pH 7.4): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4, and stored at −80 °C.

Cloning procedures

The genomic sequences encoding the putative nitrilase from *Py. abyssi* (GenBank Accession No. AJ248287), *Pyrococcus furiosus* (GenBank Accession No. AE01021), *Pyrococcus horikoshii* (GenBank Accession No. AP000003), *Sulfolobus solfataricus* (GenBank Accession No. AE006675), *Rh. rhodochrous* J1 (GenBank Accession No. D67026), and *Aeropyrum pernix* (GenBank Accession No. AP000064) were obtained from the NCBI data bank. The chromosomal DNA from *Py. abyssi* was isolated using kits obtained from Qiagen GmbH (Hilden, Germany) and following the manufacturer's instructions. The gene coding for the nitrilase from *Py. abyssi* (*nit-30*) was amplified by PCR from the genomic DNA using the following primer pairs: Nit-30 (*Nde*I) CATATGGTGAAAGTCGCCTAT and Nit-30 (*Bam*HI) GGATCCTCATCTAAAGTAGTA, that include the restriction sites *Nde*I and *Bam*HI at the 5′ and 3′-ends of *nit-30*, respectively. The gene amplification was carried out using the "hot start" conditions: denaturation at 94 °C for 5 min, followed by the addition of *HiFi* DNA polymerase (Roche, Germany); each of the following 30 cycles included 94 °C for 1 min, 55 °C for 1 min, 72 °C for

[1] *Abbreviations used:* IPTG, isopropyl-β-D-thiogalactopyranosid; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; CD, circular dichroism spectroscopy; DSC, differential scanning calorimetry.

1 min 30 s, and the final step was 72 °C for 15 min. The resulting fragments were ligated into the expression vector pET11a and transformed into the expression hosts *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS (Novagen, Germany). The genotype of the host cells is fully described in the manual of Novagen for the pET-cloning system. All cloning experiments were performed according to the Sambrook's molecular cloning manual [25]. The correct cloning of the nitrilase gene was confirmed in all cases both by sequencing and by restriction enzyme analysis. Plasmid DNA was isolated using plasmid isolation kit (Qiagen, Hilden, Germany).

Protein purification

Enzyme purification procedures were performed at room temperature. The bacterial pellet from 5 L of *E. coli* culture was resuspended in a standard buffer (20 mM K-phosphate, pH 7.4) and sonicated on ice with 100 pulses at 50% duty for 5 min. The cell extract was clarified by centrifugation in a SS-34 Sorvall rotor at 48,745*g* for 20 min at 4 °C. The soluble supernatant was heat denatured at 80 °C for 10 min, centrifuged at 20,595*g* for 10 min, and the non-precipitated protein fraction was directly applied to a 25 ml Q-Sepharose Fast Flow column (Pharmacia, Sweden) equilibrated with the standard buffer. The nitrilase was eluted with a standard buffer using a linear gradient (0–1 M NaCl) with a flow rate of 60 ml/h. The target protein was eluted with 0.55–0.65 M NaCl. Protein fractions containing the nitrilase activity were analyzed by polyacrylamide gel electrophoresis (PAGE), pooled, and desalted with Sephadex PD-10 column (Pharmacia, Sweden). Protein concentration was determined using bovine serum albumin (BSA) as standard [26].

Activity assay

Nitrilase activity was measured using malononitrile as substrate in a total reaction volume of 500 µl in a capped tube and incubated at various temperatures. The standard assay consists of 30–60 µg of the purified recombinant nitrilase in 20 mM K-phosphate, pH 7.4, 1 mM dithiothreitol (DTT), and 10 mM of substrate; incubation was performed at 80 °C for 1 h. Control experiments were run to measure the abiotic decomposition of the substrate. All measurements were performed in triplicate. The reaction products were analyzed by HPLC.

Electrophoresis

Native PAGE was performed in precast 4–20% gradient Tris–glycine gels from Novex (Invitrogen, The Netherlands). Denatured SDS–PAGE (0.1% SDS–10% PAGE) [27] was used to estimate the molecular mass of the subunits using standard proteins (Pharmacia, Sweden): phosphorilase bc (94 kDa), bovine albumin (67 kDa), chicken ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine α-lactalbumin (14.4 kDa). The protein bands were detected with Coomassie brilliant blue R-250.

Molecular mass determination

The molecular mass of the recombinant nitrilase was determined by analytical gel filtration on a Superdex 200 (Pharmacia, Sweden) column using 20 mM K-Na phosphate buffer with pH 7.4, containing 150 mM NaCl and 1 mM DTT. The column was calibrated with the following standards (Sigma–Aldrich, Germany): blue dextran (2000 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

Isoelectric point determination

Isoelectric focusing was performed using Novex precast polyacrylamide gels (pH 3–10) and a Novex electrophoresis chamber following instructions of manufacturer (Novex, Invitrogen, The Netherlands). Serva IEF marker with pH 3–10, was used as standard.

Influence of pH and temperature

The influence of pH on the nitrilase activity was measured under the standard assay conditions using the following buffers: 100 mM McIlvaine buffer (0.2 M Na_2HPO_2 and 0.1 M citric acid) for pH 4.5–5.5 and 100 mM K-Phosphate buffer for pH 6–8.5. The pH value of each buffer was checked at 80 °C. The temperature dependence of the recombinant nitrilase was estimated under the standard conditions (pH 7.2) in the range of 30–90 °C. The thermostability of the enzyme was studied by incubation at 70–90 °C for appropriate periods of time. After incubation, all samples were analyzed for the nitrilase activity under the standard conditions.

Michaelis–Menten constants were determined for malononitrile by calculating the initial rate of substrate hydrolysis in the range of 0.5–15 mM. All experiments were done in triplicate.

Effect of metal ions and other reagents

The effect of various reagents and metal ions (Ca^{2+}, Cu^{2+}, Cr^{3+}, Fe^{2+}, Ag^{+}, and Hg^{2+}) on the enzymatic activity was tested at a final concentration of 1–5 mM. The influence of the solvents acetone, ethanol, methanol, and ethylene glycol was tested at 10–50% (v/v). Samples containing the protein (10 µg) and a reagent (1 mM) were preincubated for 1 h at room temperature, then 10 mM malononitrile was added and the standard activity was measured as described above.

Analytical methods

The products of the hydrolysis of nitriles were analyzed by HPLC using an Aminex HPX-87H column (Bio-Rad,

Germany). Each injected sample (10 μl) was eluted at 50 °C with 5 mM H_2SO_4 at a flow rate of 0.7 ml/min and products were detected at 210 nm. Ammonia concentration was determined using a commercially available ammonia kit based on the phenol-hypochloride method (Spectroquant 114752; Merck, Darmstadt, Germany). The ammonia liberated was determined spectrophotometrically using ammonium chloride as standard.

Circular dichroism spectroscopy

Circular dichroism spectroscopy (CD) measurements were conducted using a JASCO-715 spectropolarimeter with a Peltier type cell holder, which allows for temperature control at the Center for Crystallography of Macromolecules at NCSR "Demokritos." Wavelength scans in the far (190–260 nm) and the near (260–340 nm) UV regions were performed in Quartz SUPRASIL (HELLMA) precision cells of 0.1 and 1 cm path length, respectively. Each spectrum was obtained by averaging 5–8 successive accumulations with a wavelength step of 0.2 nm at a rate of 20 nm/min, response time 1 s and band width 1 nm. Buffer spectra were accumulated and subtracted from the sample scans. The absorption spectra were recorded selecting the UV (single) mode of the instrument.

Differential scanning calorimetry

For the high-sensitivity calorimetric measurements the VP differential scanning calorimetry (DSC) calorimeter was employed (Microcal, Northampton, USA). Protein concentrations used in the DSC studies varied between 0.8 and 1.2 mg/ml. Protein samples and buffer reference solutions were properly degassed and carefully loaded into the cells to avoid bubble formation. Four to five reference scans with buffer filled cells (sample and reference cell volume is 0.523 ml) preceded each sample run to achieve near perfect baseline repeatability. A typical DSC experiment consisted of a heating scan at a programmed heating rate followed by a second heating scan, which probed the irreversibility of the transitions under study. Whenever needed, the difference in the heat capacity between the initial and final states was modeled by a sigmoid chemical baseline. The calorimetric data were analyzed via non-linear least squares fitting procedures of the ORIGIN 5.0 software.

Results and discussion

Identification of nitrilase genes in archaeal genomes

Most of the known nitrilases were obtained from bacterial species, isolated from environmental samples by enrichment cultures with nitriles as sole source of nitrogen [28]. This enrichment strategy resulted usually in the isolation of a rather restricted group of bacteria, such as Rhodococci. Another approach to detect nitrilase-coding genes is the use of gene similarity among the genomes deposited in the databases. Thus a putative nitrilase gene was identified in the genome of the cyanobacterium *Synechocystis* sp. PCC6803 and successfully cloned and overexpressed in *E. coli* [29]. The characterization of the enzyme revealed nitrilase activity on various aromatic and aliphatic nitriles. Due to the constant demand for new nitrilases with high stability towards temperature, substrate, and product concentrations the last strategy was used to identify putative nitrilase genes within the genomes of hyperthermophilic archaea, namely *Py. abyssi*, *Py. furiosus*, *Py. horikoshii*, *Ae. pernix*, and *S. solfataricus*. Comparison of the gene sequence of the nitrilase from *Rh. rhodochrous* JI with the genomes of extremophilic archaea mentioned above showed no significant similarity among the sequences on the nucleotide level. Comparison on the amino acid level indicated several amino acid sequences with nearly marginal homology of 28% (Table 1). The size of the putative nitrilases varies from 300 to nearly 400 amino acids (data not shown). The conserved regions were detected at the positions of amino acids 48, 131, and 166. The cysteine residue (166 amino acid), which is involved in the currently known catalytic mechanism [30], is absolutely conserved in all compared sequences (Fig. 1). Additionally, the N-terminal signature of already described microbial nitrilases VKVA-x-VQ [31,32] was also identified in the sequences of the putative nitrilases from the archaeal genomes. This observation was the first indication for the existence of potential nitrilase genes in the examined archaeal genomes.

Amino acid composition

A comparison of amino acid composition of the thermostable nitrilase from *Py. abyssi* and the nitrilase from the mesophile *Rh. rhodochrous* J1 showed (Table 2) that the number of hydrophobic amino acids (+7.4%) and charged

Table 1
Composition of amino acid sequence identities of the putative nitrilases from *Pyrococcus abyssi* (GenBank Accession No. AJ248287), *Pyrococcus furiosus* (GenBank Accession No. AE01021), *Pyrococcus horikoshii* (GenBank Accession No. AP000003), *Aeropyrum pernix* (GenBank Accession No. AP000064), and *Sulfolobus solfataricus* (GenBank Accession No. AE006675) with the nitrilase from *Rhodococus rhodochrous* J1 (GenBank Accession No. D67026)

Organism	*Py. abyssi*	*Py. furiosus*	*Py. horikoshii*	*Ae. pernix*	*S. solfataricus*
Rh. rhodochrous	28/48	26/47	26/46	n	25/46
Py. abyssi	—	84/93	86/95	28/41	32/54
Py. furiosus		—	82/92	28/41	32/55
Py. horikoshii			—	30/43	32/55
Ae. pernix				—	n

Similarity: $X/Y = X\%$ identity/$Y\%$ homology; n, no similarity.

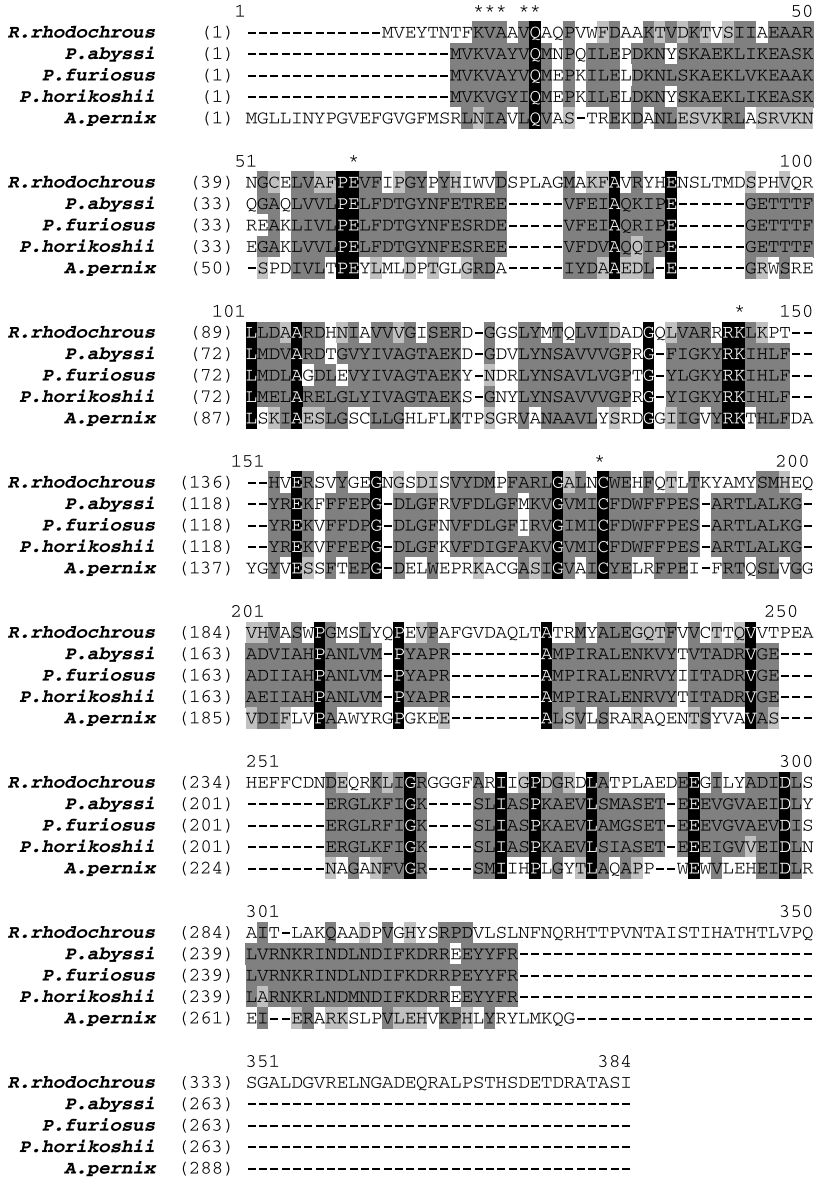

Fig. 1. Amino acid alignment of the nitrilase from *Rh. rhodochrous* J1 (GenBank Accession No. D67026) and the putative nitrilases from *Py. abyssi* (GenBank Accession No. AJ248287), *Py. furiosus* (GenBank Accession No. AE01021), *Py. horikoshii* (GenBank Accession No. AP000003), and *Ae. pernix* (GenBank Accession No. AP000064) (Vector NTI 5.3 program). AB: no homology CD: weak similarity of amino acids; XY: conserved amino acids; PM: identical amino acids; *: nitrilase catalytic signature (the triade Glu(E)-Lys(K)-Cys(C)) and N-terminal signature (KVA-x-VQ).

Table 2
Amino acid composition of the nitrilases (%)

Amino acid	*Pyrococcus abyssi* (T_{opt}: 80 °C)	*Rhodococcus rhodochrous* J1 (T_{opt}: 30 °C)
Charged (RKHDE)	29.3	24.4
Acidic (DE)	15.2	12.6
Basic (KR)	13.4	8.0
Polar (NCYST)	15.7	20.3
Hydrophobic (AILFWV)	51.9	44.5
Aromatic (FWY)	11.9	8.3

amino acids (+5%) is higher in the thermostable nitrilase. Also an increased amount of acidic (+2.6%) and basic (+5.4%) amino acids was observed. This could indicate that the formation of ionic pairs and networks between the amino acids may improve enzyme stability [33,34]. It is interesting to note that most of the hydrophobic residues are located in the internal part of the archaeal nitrilase; this is also known to stabilize structure of thermoactive enzymes [35]. Additionally, the archaeal nitrilase contains more aromatic amino acids (+3.6%). The interactions between these amino acids might have protein-stabilizing effects [36]. The disulfide-bridges known from the literature as stabilizers of proteins [37,38] are not present in the nitrilase from *Py. abyssi*. The nitrilase has only one cysteine residue, which is involved in the catalytic center. It can be speculated that the inactivation of the enzyme at 90 °C (half-life for 6 h) can be attributed to the oxidation of

cysteine in the active center. Thus, a Zi-containing aminoacylase, which has no cysteine in active site, is still 100% active even after incubation for 7 h at 100 °C [39]. In general, proteins from hyperthermophiles have a lower amount of cysteine than their mesophilic counterparts [40–42]. The nitrilase from *Rh. rhodochrous* J1 with four cysteine residues is not active at temperatures above 60 °C.

Cloning and purification

All putative nitrilase genes identified in the archaeal genomes were amplified from the chromosomal DNA of the hyperthermophilic archaea and cloned in *E. coli* as described in Material and methods. Among the overexpressed proteins nitrilase was detected only in crude extracts of the recombinant *E. coli* harboring nitrilase from *Py. abyssi*. The recombinant protein expressed under the T7 promoter was soluble and active for further biochemical and biophysical analysis. The recombinant nitrilase was purified with a specific activity of 0.14 U/mg using a two-step purification procedure including heat treatment followed by ion-exchange chromatography. A total of 3.1 mg nitrilase was purified from 3 g of cell (wet weight) with a purification factor of 24-fold and a yield of 88% (Table 3).

Physicochemical properties

The purified enzyme migrated as a single band in SDS–PAGE (10 μg of protein) with a molecular mass of 30 kDa (Fig. 2A). By gel filtration on Superdex 200 column (data not shown) and PAGE under native conditions, the molecular mass of the native protein was estimated to be 60 kDa (Fig. 2B), indicating that the native enzyme exists as a dimer. The subunit molecular mass and the dimeric structure of the native archaeal nitrilase is close to that from mesophilic bacteria *Klebsiella ozaenae* (38 kDa), *Ps. fluorescens* DSM 7155 (40 kDa), and actinomycete *Rh. rhodochrous* J1 (41 kDa). Other known nitrilases are composed of more than two subunits, such as the enzyme from *Nocardia* sp. NCIB 11216 with a molecular mass of 560 kDa (46 kDa/subunit) [18,43]. The nitrilase from *Rh. rhodochrous* K22 is a 650-kDa protein with fifteen or sixteen 41-kDa subunits [44]. The p*I* of the recombinant archaeal nitrilase was determined to be 5.3 (Fig. 2C). This value is close to the theoretically calculated isoelectric point, using the NIT 5.3 DNA analysis program, and to the p*I* value 5.6 of the regiospecific nitrilase from *Rh. rhodochrous* J1 [21].

As expected, the enzyme derived from the hyperthermophilic microorganism is active at elevated temperatures with a maximum of apparent activity at 80 °C. At 60 and 90 °C, the enzyme exhibits more than 10% of its maximal activity. Since the organism grows optimally at 100 °C, it is highly likely that the enzyme is most flexible at this temperature. Nitrilases are intracellular enzymes; therefore it is not surprising, that the recombinant enzyme shows high activity within the pH range of 4.5–8.5 with an optimum at pH 7.4. This is comparable to the values described for the nitrilases from *Alcaligenes faecalis* JM3 [17], *B. pallidus* Dac521 [23], and several *Rhodococcus* strains [16,19]. The only nitrilase, which is optimally active at pH 9.0 was isolated from *Ps. fluorescens* DSM 7155 [28]. The recombinant nitrilase from *Py. abyssi* exhibits high thermostability. After 25 h of incubation at 70 °C the recombinant nitrilase has nearly 50% of residual activity, while at 80 and 90 °C the half-life of the enzyme is 9 and 6 h, respectively. These are the highest values described so far for microbial nitrilases. The optimal temperature reported for a nitrilase from the thermophile *B. pallidus* Dac521 is 65 °C and the half-life of this enzyme is <3 min at 80 °C [23], compared to the half-life of 9 h at 80 °C for the archaeal enzyme. Most of nitrile-degrading enzymes isolated from mesophiles are often unstable at the temperatures optimal for the growth of microorganisms [6]. The *Rh. rhodochrous* J1 nitrilase retains only 7% of its initial activity when incubated for 1 h at 50 °C [45]. It is interesting to note that mesophiles (e.g., *Rhodococcus* sp.) as well as the hyperthermophile *Py. abyssi*, isolated from deep-sea, are able to produce nitrile-degrading enzymes [46].

The thermal stability of recombinant nitrilase was also studied by employing circular dichroism spectroscopy (CD) and differential scanning calorimetry (DSC) at concentrations between 0.4 and 0.5 mg/ml. The recombinant nitrilase has shown a remarkable thermal stability, since the enzyme is resistant, even to partial denaturation, at temperature up to 90 °C (Fig. 3A), while the T_m, determined by DSC, is 112.7 °C (Fig. 3B). Heating the enzyme up to 120 °C it is irreversibly denatured. Therefore, this nitrilase can be considered as the most thermostable enzymes described so far.

Inhibitors, metal ions, and other reagents

Various thiol reagents, at a concentration of 5 mM, completely inhibit the activity of the recombinant nitrilase (Table 4a) as already demonstrated for other nitrilases [6]. These data indicate the involvement of the highly

Table 3
Purification of the recombinant nitrilase from *Pyrococcus abyssi*

Step	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)[a]	Yield (%)	Purification fold
Crude extract[b]	84	500	5.95	100	1
Heat treatment	17.5	510	29.14	102	4.89
Q-Sepharose	3.1	440	141.9	88	23.85

[a] One unit of nitrilase catalyzes the formation of 1 μmol of malonic acid per min per mg of protein under the standard conditions.
[b] After the growth at 37 °C, a 1-liter of culture was centrifuged (3 g of cells [wet weight]); the cells were washed twice with standard buffer (pH 7.4) and disrupted by sonication.

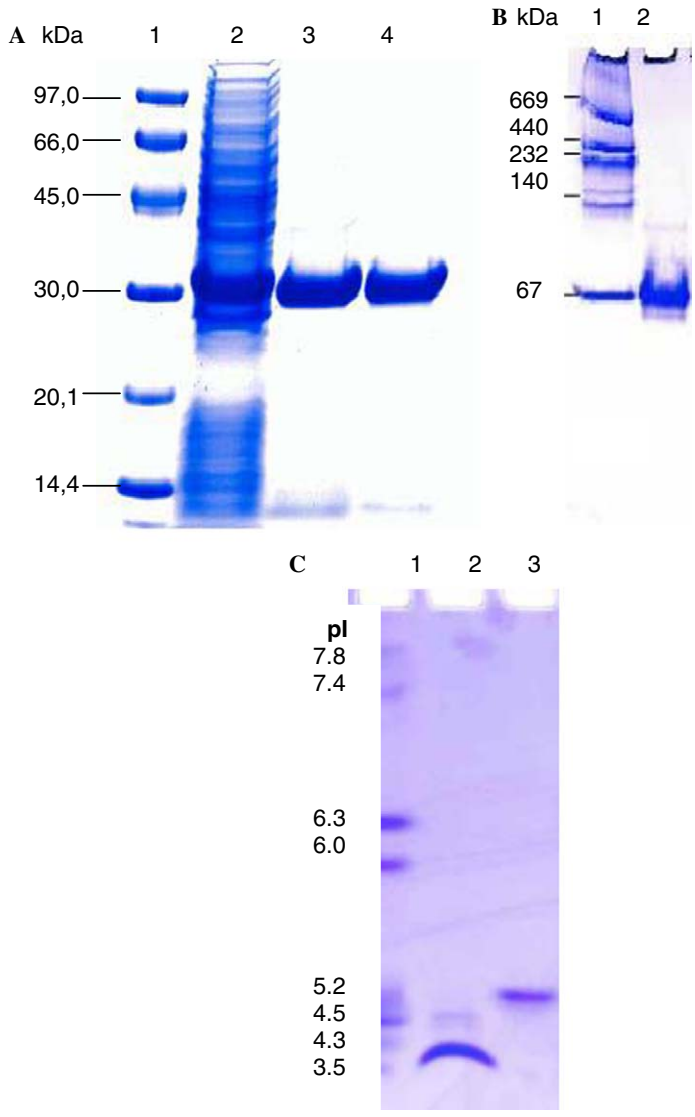

Fig. 2. (A) Electrophoretic analysis (SDS–PAGE) of samples containing nitrilase activity from various purification steps. Lane 1, low molecular weight standard proteins; lane 2, *E. coli* crude extract; lane 3, pool of proteins after the heat treatment; lane 4, Q-Sepharose pool. In each lane 10 μg of protein was applied. The proteins were stained with Coomassie blue. (B) Electrophoretic analysis (Native PAGE) of the recombinant nitrilase. Lane 1, standard proteins; lane 2, 10 μg of the purified enzyme. (C) Isoelectric focusing PAGE of the recombinant nitrilase. Lane 1, p*I* of standard proteins; lane 2, standard protein with p*I* 4.3; lane 3, recombinant nitrilase with estimated p*I* 5.3.

conserved cysteine residue in the catalytic mechanism (Fig. 1). This is also supported by the complete inhibition of the enzymatic activity by the cysteine-bound metals Ag^{+} and Hg^{2+} and reagents, such as *p*-chloromercuribenzoate and *p*-hydroxymercuribenzoate. Iodacetamide and iodacetate at a concentration of 1 mM inactivate the enzyme significantly. These results support the proposed mechanism for nitrilases [3,18], which includes a nucleophilic attack on the nitrile carbon atom by a thiol group at the active site of the enzyme. The resulting enzyme-iminothiol ester is hydrolyzed via the tetrahedral amino-hydroxy-thioester intermediate to the thiol ester, which is further hydrolyzed to the acid.

Other metal ions have no significant effect on the nitrilase activity. The negative influence of the iron ions could be due to a redox-effect or due to the formation of an iron-complex with the thiol group of the enzyme [18]. EDTA has no influence, suggesting that additional divalent cation(s) are not required for the activity of the thermoactive nitrilase. In general, nitrilases are not metal dependent enzymes. The presence of reducing agents, such as DTT, L-cysteine and 2-mercaptoethanol, at a concentration of 1 mM, were found to activate the nitrilase (Table 4a), indicating the important role of sulfhydryl groups in enzyme action. All tested organic solvents inhibit the nitrilase activity completely at concentrations higher than 10% (Table 4b), which is in agreement with the data obtained for other microbial nitrilases. The presence of methanol decreased the enzymatic activity rapidly, possibly due to the blocking of the active site of the enzyme. In contrast to the nitrilase from

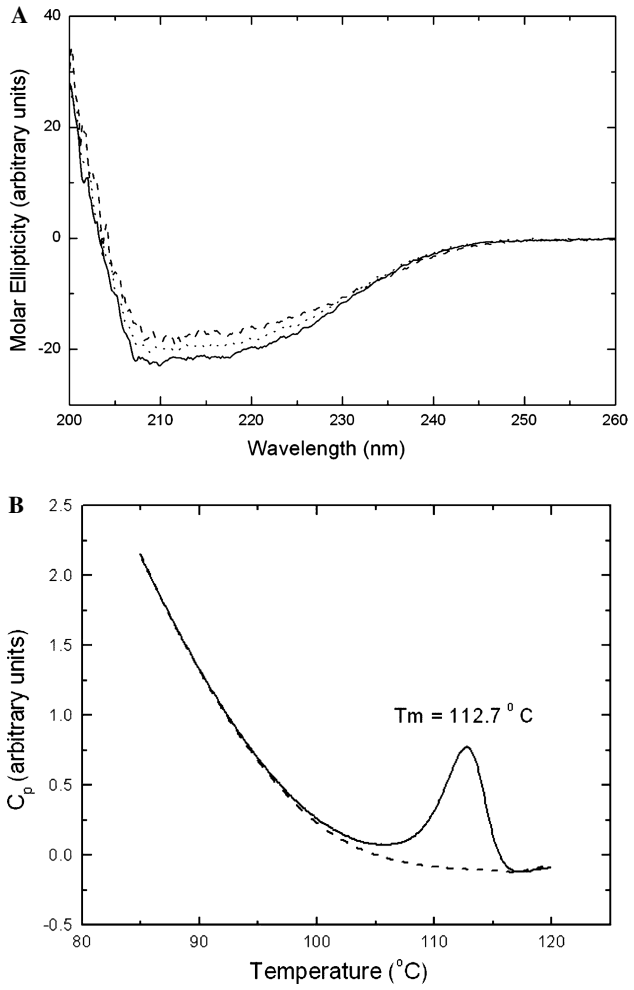

Fig. 3. (A) Far UV CD spectra for nitrilase from *Pyrococcus abyssi* at various temperatures below the melting transition: At 20 °C (solid line), at 90 °C (dash line), and 20 °C after cooling from 90 °C (dotted line). (B) DSC profile for the thermal denaturation of nitrilase *Pyrococcus abyssi* at heating rate $u = 1.5$ K/min, showing an irreversible endothermic Cp peak at T_m 112.7 °C (solid line). The second DSC heating run is also displayed (dash line).

Table 4a
Influence of various reagents, ions, and chelating agents on the recombinant nitrilase from *Pyrococcus abyssi*

Compounds	Relative nitrilase activity (%)	
	1 mM	5 mM
None	100	100
p-Chloromercuribenzoate	0	0
DTNB (Dithiobisnitrobenzoic acid)	43	0
p-Hydroxymercuribenzoate	0	0
Iodoacetamide	81	0
Iodoacetate	79	0
L-Cysteine	110	73
DTT	123	50
Phenyl hydrazine	69	53
2-Mercaptoethanol	100	71
Ag^{+}	0	0
Ca^{2+}	80	75
Cr^{3+}	82	60
Fe^{2+}	75	40
Hg^{2+}	0	0
EDTA	100	95
NaN_3	100	97

Samples containing the protein (10 μg) and a reagent (1–5 mM) were preincubated for 1 h at 22 °C, then the standard activity was measured by incubating the samples with malononitrile (10 mM) in 20 mM phosphate buffer at 80 °C for 1 h.

Table 4b
Influence of various solvents (10–50%) on the recombinant nitrilase from *Pyrococcus abyssi*

Solvent	Remaining nitrilase activity (%)		
	10%	30%	50%
None	100	100	100
Acetone	0	0	0
Ethanol	90	43	0
Methanol	10	0	0
Ethylene glycol	120	70	20

Samples of the purified nitrilase (10 μg) were preincubated with the above reagents at 22 °C for 1 h, then the standard activity was measured by incubating the samples with malononitrile (10 mM) in 20 mM phosphate buffer at 80 °C for 1 h.

Py. abyssi, ethanol and acetone activate the subunit association of the nitrilase from *Rh. rhodochrous* J1 resulting in an increase of the enzymatic activity [20]. Ethylene glycol, which is generally used as protein stabilizer, at 10% activates the enzyme. Similar data have been reported for the nitrilase from the thermophile *B. pallidus* Dac521, where ethylene glycol at concentrations between 20 and 30% causes enzyme activation [23].

Substrate spectrum

Microbial hydrolysis of aromatic nitriles has been known for many years, but the description of nitrilases that convert aliphatic nitriles is relatively new and includes those from *Rh. rhodochrous* K22 [16,44], *Comamonas testosteroni* [47], and *A. facilis* 72W [22]. The purified thermoactive nitrilase from the archaeon *Py. abyssi* shares features of this group. The recombinant nitrilase has a narrow spectrum and is highly specific towards aliphatic dinitriles. Fumaronitrile and malononitrile were found to be the best substrates for the nitrilase from *Py. abyssi* with K_m of 9.48 and 3.47 mM, respectively. Malononitrile at a concentration above 12 mM has a strong inhibitory effect on the archaeal enzyme. Compounds with a nitrile group bound to an aromatic ring or amino acid were not hydrolyzed by the recombinant nitrilase. The ability of the archaeal nitrilase to hydrolyze fumaronitrile 10 times faster than malonitrile was observed in the case of the bacterial nitrilase from *A. facilis* 72W [22]. Both compounds were converted by the *Py. abyssi* nitrilase to the monoacid mononitrile, suggesting that this enzyme, as well as the enzymes from *Ps. fluorescens* [15] and *A. facilis* 72W [22], are regioselective. The nitrilase from *Py. abyssi* is an attractive candidate for industrial application, especially for the conversion of dinitriles to mononitriles. In general, it is difficult or impossible to obtain mononitriles chemically, the process is highly dependent on the substrate nature [48].

A biotechnological process has been described using the whole-cells of *Rh. rhodochrous* NCIB 11216. Fumaronitrile and succinonitrile were converted completely to the corresponding mononitriles [49,50]. Another nitrilase from *Rh. rhodochrous* K22 hydrolyzes glutaronitrile to the corresponding mononitrile without the formation of glutaric acid [16]. The extreme stability and regioselectivity of the nitrilase from *Py. abyssi* opens up the possibility for the application of the enzyme in industry.

The present study demonstrates, that the hyperthermophilic archaeon *Py. abyssi* harbors a gene encoding nitrilase, which could be essential for growth on nitriles or detoxification of these compounds. Under tested conditions the strain, however, is unable to grow on nitriles and nitrilase activity could not be detected in cell extracts. It is very likely that the nitrilase gene has no physiological function in this archaeon. Further investigations, including crystallograhy and NMR studies, will add more knowledge on the mechanism involved in protein stabilization.

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