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A Novel Aryl Acylamidase From *Nocardia farcinica* Hydrolyses Polyamide

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ABSTRACT: An alkali stable polyamidase was isolated from a new strain of *Nocardia farcinica*. The enzyme consists of four subunits with a total molecular weight of 190 kDa. The polyamidase cleaved amide and ester bonds of water insoluble model substrates like adipic acid bishexylamide and bis(benzoyloxyethyl)terephthalate and hydrolyzed different soluble amides to the corresponding acid. Treatment of polyamide 6 with this amidase led to an increased hydrophilicity based on rising height and tensiometry measurements and evidence of surface hydrolysis of polyamide 6 is shown. In addition to amidase activity, the enzyme showed activity on *p*-nitrophenylbutyrate. On hexanoamide the amidase exhibited a K_m value of 5.5 mM compared to 0.07 mM for *p*-nitroacetanilide. The polyamidase belongs to the amidase signature family and is closely related to aryl acylamidases from different strains/species of *Nocardia* and to the 6-aminohexanoate-cyclic dimer hydrolase (EI) from *Arthrobacter* sp. KI72.

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Introduction

The most common synthetic polyamides (PAs) are polyamide 6 (Nylon 6, Perlon) and polyamide 6.6 (Nylon 6.6). Nylon filaments are used as yarns for textile, industrial and carpet applications and a growing demand was reported especially for industrial and textile applications (Saurer Management AG, 2007). Nylon based textiles show the great disadvantage to be uncomfortable to wear and difficult to finish due to their hydrophobicity. Similarly, fouling of PA based ultrafiltration membranes by proteins and other biomolecules is due to the low hydrophilicity and increases the energy demand for filtration and requires cleaning with aggressive chemicals or replacement (Asatekin et al., 2007; Kim et al., 2007; Li et al., 2007; Qiao et al., 2007). Therefore, enhancement of the hydrophilicity of nylon is a key requirement for many applications and can be achieved by using plasma treatment (De Geyter et al., 2007; McCord et al., 2002; Tusek et al., 2001) or more recently by using enzymes. Enzymatic treatment of nylon was carried out with enzymes from different enzyme classes (i.e., oxidoreductases and hydrolases) and is specific to the polymer surface (Fujisawa et al., 2001; Klun et al., 2003; Negoro, 2000). When compared to plasma treatment, enzyme modification of polyamides requires less energy and is not restricted to planar surfaces.

Treatment of polyamide with hydrolases showed potential for targeted surface modification without changes of bulk properties. Limited surface hydrolysis leads to generation of polar groups without significant solubilization of oligomers. In contrast, treatment with peroxidases seems to be difficult to control leading to substantial damage of fibers (Guebitz and Cavaco-Paulo, 2008). Most previous studies on enzymatic polyamide modification have used commercial proteases or well known cutinases while the large enzyme diversity of nature has not been exploited so far for this purpose. This is the first report on a ''polyamidase'' isolated from nature. In a preliminary screening a bacterium hydrolyzing polyamides was isolated from soil samples and identified as *Nocardia farcinica* (Fischer-Colbrie et al., 2004;

Heumann et al., 2006). *Nocardia* spp. are widespread soil bacteria, aerobic and saprophytic actinomycetes (Wu et al., 2006). *Nocardia* spp. produce bioactive molecules and enzymes which are of industrial importance (Ishikawa et al., 2004).

The aim of this work was to study a novel amidase isolated from *N. farcinica* related to its potential to hydrolyze water insoluble polyamide oligomers and polyamide 6 thereby increasing surface hydrophilicity.

Materials and Methods

Materials

Acetonitrile and methanol were HPLC quality and purchased by Roth (Carl Roth GmbH, Karlsruhe, Germany). Water was distilled twice. All other chemicals were of analytical grade and supplied by SIGMA (Vienna, Austria). The model substrates for polyamide 6.6 adipic acid bishexylamide and for polyethylene terephthalate (PET) the model substrate bis(benzoyloxyethyl) terephthalate were synthesized as described previously (Fischer-Colbrie et al., 2004; Heumann et al., 2006).

Microorganisms and Cultivation Conditions

Several bacteria isolated from soil samples as described previously (Fischer-Colbrie et al., 2004) were found to hydrolyze adipic acid bishexylamide (model substrate for polyamide 6.6 (Heumann et al., 2006). Out of these, we selected a strain with highest activity on this substrate for the current study. This strain was identified as *N. farcinica* at DSMZ culture collection (German Collection of Microorganisms and Cell Cultures) using standard methods including 16S rRNA comparison, morphology, physiology, fatty acid pattern and the length of mycolic acids and is deposited under the number 05-253.

N. farcinica was inoculated into a cultivation broth containing $2.5\,g\,L^{-1}$ KH_2PO_4, $3.0\,g\,L^{-1}$ K_2HPO_4, $2.0\,g\,L^{-1}$ NH_4Cl, $0.3\,g\,L^{-1}$ $MgSO_4 \cdot 7H_2O$, $5\,mL\,L^{-1}$ trace element solution with pH 7.0 and $0.8\,g\,L^{-1}$ adipic acid bishexylamide (model substrate for polyamide 6.6 (Heumann et al., 2006) as the only carbon source. The trace element solution consisted of $2,500\,mg\,L^{-1}$ Na_2EDTA, $100\,mg\,L^{-1}$ $ZnSO_4 \cdot 7H_2O$, $30\,mg\,L^{-1}$ $MnCl_2 \cdot 4H_2O$, $300\,mg\,L^{-1}$ H_3BO_3, $200\,mg\,L^{-1}$ $CaCl_2 \cdot 6H_2O$, $10\,mg\,L^{-1}$ $CuSO_4 \cdot 2H_2O$, $900\,mg\,L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, $30\,mg\,L^{-1}$ $Na_2SeO_3 \cdot 5H_2O$, $1,000\,mg\,L^{-1}$ $FeSO_4 \cdot 7H_2O$ which is a modified version of Meyer and Schlegel (1983). The cultivation was carried out in 250 mL baffled Erlenmeyer flask containing 100 mL medium on an orbital shaker (125 rpm) at $30°C$ for 7 days. After 7 days the cells were harvested and washed three times with phosphate buffer (50 mM, pH 7). Cells were broken using a French press and then centrifuged at $33,000g$ at $4°C$ for 1 h. The supernatant was stored after sterile filtration at

$-18°C$. The protein content of enzyme solutions was measured according to Lowry et al. (1951).

Cultures of the isolated strain were maintained on agar plates with the same composition as the liquid medium and additionally $12\,g\,L^{-1}$ agar-agar at $4°C$ and in a glycerol stock at $-70°C$.

Enzyme Assays

Esterase Activity

Esterase activity was measured using *p*-nitrophenylbutyrate as a substrate as previously described (Heumann et al., 2006). The activity was calculated in units, where 1 unit is attributed to the amount of enzyme required to hydrolyze $1\,\mu mol$ of substrate per minute under the given assay conditions.

Protease Activity

Protease activity was determined using azocasein as substrate as previously described (Heumann et al., 2006). The activity was calculated in units where 1 unit was attributed to the amount of enzyme required to produce the absorbance change of 1.0 in 1 cm cuvette under the conditions of the assay per minute (Heumann et al., 2006).

Amidase Activity

The assay of amidase activity was carried out in a reaction mixture ($320\,\mu L$) containing $200\,\mu L$ potassium phosphate buffer (50 mM, pH 7.0), $20\,\mu L$ of a $430\,\mu M$ substrate solution and $100\,\mu L$ enzyme solution or $100\,\mu L$ buffer as a blank. The reaction was carried out at $30°C$ for 60 min and stopped with $32\,\mu L$ 10% trichloroacetic acid. After centrifugation at $16,000g$ for 5 min, the released ammonia was determined colorimetrically using the phenol/hypochlorite method (Cramp et al., 1997). Therefore, $100\,\mu L$ reaction mixture were added to $350\,\mu L$ hypochlorite solution together with $350\,\mu L$ phenol reagent. The absorbance was read at 625 nm after 15 min incubation at $50°C$. The amount of ammonia released was determined from a standard curve.

Aryl Acylamidase Activity

Aryl acylamidase activity was measured using *p*-nitroacetanilide as a substrate. The assay mixture consisted of 1 mL of a potassium phosphate buffer (50 mM, pH 7.0), $100\,\mu L$ of the enzyme solution and $10\,\mu L$ of the substrate solution (25 mg of *p*-nitroacetanilide dissolved in 1 mL DMSO and $30\,\mu L$ Triton X 100). The increase of the absorbance at 405 nm which indicates an increase of *p*-nitroanilide due to hydrolysis of the substrate was measured at $25°C$ using a spectrophotometer type Hitachi U 2001. A blank was measured using $100\,\mu L$ buffer instead of sample. The activity was calculated in units, where 1 unit is attributed to

the amount of enzyme required to hydrolyze 1 μmol of substrate per minute under the given assay conditions.

Transacylase Activity

The qualitative determination of transacylase activity (Fournand et al., 1998) was carried out in a reaction mixture (300 μL) containing 100 μL of 430 μM hexanoamide solution, 100 μL of 2 M hydroxylamine solution pH 7 and 100 μL enzyme solution. The reaction was carried out at 50°C for 30 min. To detect activity, 100 μL of this incubation solution was added to 100 μL 0.6 M ferrum(III) chloride in 1 M HCl. The blank was carried out as described above but instead of enzyme solution phosphate buffer (50 mM, pH 7) was used. Enzyme activity was indicated by a color change of the solution from yellow to red.

Polyamidase and Polyesterase Activity

Twenty milligrams of adipic acid bishexylamide or bis-(benzoyloxyethyl) terephthalate were treated with 0.6 mL enzyme solution in Eppendorf tubes and shaking at 750 rpm at 30°C. After 6 h the reaction was stopped by adding 10 μL conc. sulfuric acid. The samples were kept at room temperature for 15 min and then centrifuged at 16,000g (Hereaus, Biofuge primo) and filtered with syringe filter (13 mm, with 0.2 μm pore size) for HPLC use.

For analyses of adipic acid as a hydrolysis product of the PA model substrate the analytes were separated by HPLC using a reversed-phase column Discovery 15 cm (Supelco) as previously described (Heumann et al., 2006). One unit of the enzyme is attributed to the amount of enzyme that catalyzes the formation of adipic acid at the rate of 0.5 μmol min^{-1}. For analysis of the benzoic acid as hydrolysis product of the PET model substrate a reversed phase column (Source 5RPC ST 4.6/150 Amersham Pharmacia Biotech) was used. Separation was achieved by elution with 40% methanol, 10% 10 mM sulfuric acid and 50% water. A flux of 1 mL min^{-1} and a temperature of 40°C were adjusted. The amount of benzoic acid released was determined from a standard curve. One unit of the enzyme is attributed to the amount of enzyme that catalyzes the formation of benzoic acid at the rate of 1 μmol min^{-1}.

Enzyme Characterization

For testing the substrate specificity of the polyamidase the following amides were used: hexanoamide, p-nitrophenyl-butyrate, p-nitroacetanilide, butyramide, methacrylamide, propionamide, benzamide, acetamide, asparagine, mande-lamide, nicotinamide, and tearamide. Temperature and pH-optima and stabilities of polyamidase were tested with hexanoamide in 300 mM phosphate buffer (pH 7.0). The enzyme was incubated in different buffers and at different temperatures (30–70°C) for 30 min. Buffer 1 was a 100 mM citrate buffer ranging from pH 3 to 6, buffer 2 was a phosphate buffer (50 mM, pH 6–8), buffer 3 was a borate

buffer (85 mM, pH 8–13). To estimate temperature stability the enzyme was incubated in phosphate (pH 8) at 50°C for 1–120 min, at 25°C and at −18°C for 1 week.

Several compounds were tested for their inhibitor effects on the polyamidase. The assay with hexanoamide was carried out as described above but the enzyme was pre-incubated for 30 min in a phosphate buffer (50 mM, pH 7) containing the compounds. The following compounds in different concentrations were used: copper sulfate, mercury chloride, silver nitrate, hexylamine (1-aminohexane), EDTA, calcium chloride, cyanoacetamid, mangan chloride, zinc chloride, ferrum sulfate, and magnesium sulfate.

One unit of the amidase activity was attributed to the amount of enzyme required for the hydrolysis of 1 μmol of amide (corresponding to the formation of 1 μmol ammonia) per minute in 1 mL enzyme solution under the conditions of the assay.

Kinetic parameters were determined using the standard assays with different substrate concentrations. K_m and V_{max} were calculated by nonlinear analysis using the program "Origin," version 4.10.

Enzyme Purification

Ultrafiltration

Ultrafiltration was performed with centrifuge concentrators (Vivaspin 20, Sartorius) with molecular weight cut off of 100 kDa to concentrate small fraction sizes (up to 20 mL). Ultrafiltration was carried out in a centrifuge at 5,000g and at a temperature of 4°C. For volumes of up to 250 mL an Amicon$^®$ bioseparation stirred cell (Millipore, Billerica, MA) was used with ultrafiltration membranes made of poly-ethersulfone and a cut off 100 kDa (76 mm, Millipore). The filtration was carried out under 4°C and a pressure of 0.75 bar.

Chromatography

The enzyme purification was carried out with an Amersham pharmacia biotech ÄKTA purifier 900. Enzyme activity was monitored using hexanoamide and adipic acid bishexy-lamide as substrates.

Step 1: Hydrophobic interaction chromatography: The intracellular fraction (10.5 mL) was equilibrated with 1 M Na$_2$SO$_4$ and then placed on a butyl sepharose 4 Fast Flow column (26 mm × 80 mm dimension, 40 mL bed volume, 5.0 mL min^{-1} flow) equilibrated with phosphate buffer (50 mM) and 1 M Na$_2$SO$_4$ at pH 7. The elution was done with phosphate buffer (50 mM, pH 7) with gradient steps at a flow rate of 1 mL min^{-1} and the polyamidase was eluted at 60% elution puffer (1 M Na$_2$SO$_4$, pH 7). The active fractions were pooled and concentrated using ultrafiltration (100 kDa MWCO).

Step 2: Anionic exchange chromatography: The supernatant after ultrafiltration of step 1 (5.0 mL) was loaded on a Q Sepharose High Performance column (26 mm × 120 mm,

63 mL bed volume, 5.0 mL min^{-1} flow). The column was equilibrated with phosphate buffer (50 mM, pH 7) and the polyamidase was eluted at 50% elution buffer (50 mM phosphate buffer with 1 M NaCl, pH 7). The active fractions were pooled and concentrated by ultrafiltration (100 kDa MWCO).

Step 3: Size exclusion chromatography: The supernatant after ultrafiltration of step 2 (50 μL) was loaded on a Superdex S75 column (10 mm × 300 mm dimension, 24 mL bed volume, 0.5 mL min^{-1} flow) equilibrated with a solution containing 100 mM phosphate buffer and 100 mM NaCl at pH 7. The column was calibrated with protein standard kit with a molecular weight range from 29 to 700 kDa.

Gel Electrophoresis

Gel electrophoresis was performed with a Mini-PROTEAN 3 Electrophoresis System (Bio-Rad) according to the manufacturers instruction using pre-stained molecular weight markers covering a broad range of molecular weights (200–14.4 kDa). Gels were stained using Coomassie Blue R-250 for 30 min and afterwards destained in 40% ethanol and 10% acetic acid. To identify protein bands showing esterase activity, gels produced without SDS were stained with an esterase activity test according to Gudelj et al. (1998). Gels were incubated in 20 mL phosphate buffer (0.1 M, pH 7.0), 2 mL naphtyl acetate solution (12 mg mL^{-1} acetone) and 500 μL fast blue B solution (20 mg mL^{-1} water). The reaction was stopped by incubating the gel in a 10% acetic acid solution. Active proteins were detected as dark red or violet bands.

Aryl acylamidase activity was detected with a modified method of Jaganathan and Boopathy (2000). Therefore gels were soaked in 20 mL phosphate buffer (0.1 M, pH 7.0) and in 2 mL *o*-nitroacetyl anilide in 25 mg mL^{-1} DMSO for 30 min. Gels were washed once with bidestilled water and stored on ice. Then an ice cooled solution of 0.1 g sodium nitrite in 100 mL 1 M hydro chloride and 0,75 g *N*-(1-naphtyl)ethylene diamine was added and gently moved. Bands showing aryl acylamidase activity appeared in purple.

Peptide Mass Mapping

Polyamidase bands on gels produced without SDS were stained with Commassie Blue and cut off. Proteins were reduced with dithioerythritol, alkylated with iodacetamide and digested with trypsin. The resulting peptides were separated and detected on a nano-LC–MS/MS system from Agilent (1100 series NanoLC connected with Agilent 1100 MSD Ion Trap SL). For identification of the MS/MS spectra the SpectrumMill Proteomics Workbench from Agilent was used.

Surface Hydrophilization

Enzyme Treatment

Polyamide 6 tricot fabrics from Ciba, Switzerland were washed with sodium phosphate (Na$_2$HPO$_4$·2H$_2$O, 5 mM) solution for 30 min as pre-treatment to remove finishes from the surface; finally the fibers were rinsed with distilled water. Fabric samples were cut into pieces of 3 cm × 18 cm and treated in 250 mL Erlenmeyer flasks with the enzyme solutions (5 nkat mL^{-1}) for 10, 30, and 60 min at pH 8 and 30°C. Respective controls with inhibited enzymes (0.1% w/v HgCl$_2$), without enzyme and a sample treated with a silicone based finisher (Ciba, Basel, Switzerland) were prepared accordingly. Afterwards fabrics were washed with sodium carbonate (9.4 mM, pH 9.5) and distilled water (4 times) to remove adsorbed enzymes and other media impurities. Fabrics were dried at room temperature overnight. The absence of protein impurities adsorbed on the fiber surfaces was confirmed using Coomassie blue staining method comparing with a reference.

Measurement of Hydrophilicity

To measure the surface modification of synthetic polymers two different analytic methods indicating changes in hydrophilicity were used: Water absorption was determined using rising height while surface properties were additionally analyzed with tensiometry.

Rising height was measured using a method slightly modified from DIN 53924. The method consisted in suspending the fabrics on a glass rod and immersing the bottom (1 cm) of the fabrics into a water bath (distilled water). After 10 min the water level on the fabric was measured. For surface tensiometry the K 100 apparatus from Krüss (Hamburg, Germany) was used. The measurement was repeated three times and the average taken as the result.

Results and Discussion

A bacterium previously isolated from soil samples (Fischer-Colbrie et al., 2004) was found to hydrolyze adipic acid bishexylamide. The bacterium was identified by DSMZ culture collection as new strain of *N. farcinica* based on 99.8% similarity of the 16S rRNA with the type strain of DSMZ. Additionally the pattern of the fatty acids and the mycolic acids confirmed this result (data not shown). In this study, an intracellular amidase from this strain was purified and characterized as the first enzyme from this class shown to hydrolyze polyamide. The enzyme was produced in a cultivation medium containing adipic acid bishexylamide as the only carbon source.

Purification and Characterization of the Polyamidase

The polyamidase from *N. farcinica* was purified in 6 steps (Table I) up to a specific activity of 26.0 nkat mg^{-1} on

Table I. Purification of the polyamidase from *Nocardia farcinica*.

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg^{-1})	Recovery (%)
Cell free extract	59.9	62.7	1.1	100
Hydrophobic interaction chromatography	7.4	58.5	8.1	93
Ultrafiltration 100 kDa	3.8	58.3	15.4	93
Anionic exchange chromatography	0.8	20.5	27.2	33
Ultrafiltration 100 kDa	0.3	12.2	39.7	19
Size exclusion chromatography	0.01	0.2	26.0	0.4

Activity was measured with hexanoamide as substrate.

hexanoamide as substrate. This activity correlated with activity on the PA model substrate adipic acid bishexylamide and was used to follow activity during purification due to easier measurement. The specific activity decreased during the last step from 39.7 down to 26.0 nkat mg^{-1} since the enzyme partially denatured during size exclusion chromatography. The polyamidase was purified up to 40-fold to electrophoretic homogeneity (Fig. 1) with a yield of 0.1%. This was less compared to the 290-fold purification of aryl acylamidase from *Nocardia globerula* (Yoshioka et al., 1991). The enzyme was digested with trypsin and the results from the LC–MS/MS were evaluated using the SwissProt data base. The calculated molecular weight of the polyamidase subunit after tryptic digest was 50 kDa, after SDS gel electrophoresis 54 kDa. The native polyamidase had a molecular weight of 190 kDa. The polyamidase of *N. farcinica* was thus constructed of 4 subunits.

Figure 1. Native gel electrophoresis of the polyamidase from *Nocardia farcinica* after size exclusion chromatography (fraction 20 and 21). Protein bands were stained with silver. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Temperature and pH Dependences

The polyamidase had its temperature optimum at 50°C (pH 8). At these conditions the half life time of the enzyme was 35 min. At 30°C, the enzyme retained more than 80% of its activity over a pH-range from pH 6 to pH 11 with optimum activity between pH 8 and 11. After storage at 25 and −18°C for 1 week, the polyamidase only lost 5% and 18% of its activity, respectively. Amidases with similar substrate specificity (including 6-aminohexanoate oligomer hydrolases) had temperature optima between 30 and 45°C while pH optima were observed from pH 7.0 to 9.5 at 30°C. The stabilities of these enzymes were strongly dependent on the temperature and pH (Ciskanik et al., 1995; Fournand et al., 1998; Hayashi et al., 1997; Hirrlinger et al., 1996; Krieg et al., 2002; Negoro, 2000). The longest half life at room temperature reported for an amidase was 2,000 h (Hirrlinger et al., 1996).

Substrate Specificity and Inhibitors

The polyamidase hydrolysed *p*-nitroacetanilide, *p*-nitrophenylbutyrate, and various amides and esters (Table II). The highest activity was observed with *p*-nitroacetanilide and *p*-nitrophenylbutyrate. This activity is specific for aryl

Table II. Substrate specificity of the polyamidase from *N. farcinica*, compared to activity on hexanoamide (1.6 nkat mg^{-1}) set as 100%.

Substrate	Relative activity (%)
Hexanoamide	100
p-Nitrophenylbutyrate	669
p-Nitroacetanilide	288
Butyramide	156
Methacrylamide	87.8
Propionamide	76.4
Benzamide	35.2
Acetamide	20.4
Asparagine	14.5
Mandelamide	7.6
Nicotinamide	1.2
Stearamide	1.1
Proteolytic activity with azocasein	0.00
Adipic acid bishexylamide	0.13
Bis (benzoyloxyethyl) terephthalate	0.04

The experiments were carried out in three replicates and the average is given (standard deviation <8%).

acylamidases which was also reported by Yoshioka et al. (1991). High activities were obtained with butyramide, hexanoamide and propionamide (Table II). On the other hand, benzamide was hydrolysed faster than acetamide. The polyamidase also hydrolysed water insoluble substrates like adipic acid bishexylamide and bis (benzoyloxyethyl) terephthalate. However, compared to water soluble substrates the activity was rather low. Interestingly, the polyamidase did not show any protease activity while previously some proteases were found to hydrolyse PA (Miettinen-Oinonen et al., 2007; Silva et al., 2005). The polyamidase from N. farcinica showed a broad substrate specificity compared to aryl acylamidases from Rhodococcus sp. and Pseudomonas sp. (Hammond et al., 1983; Hirrlinger et al., 1996). Only the aryl acylamidase from N. globerula showed a similarly broad substrate specificity (Yoshioka et al., 1991).

The polyamidase of N. farcinica was able to catalyze the transfer of the acylgroup of hexanoamide to hydroxylamine which is common for amidases belonging to EC 3.5.1.4 (Fournand et al., 1998). On hexanoamide, the enzyme had a K_m value of 0.70 mM and a k_{cat} of 3.5 s^{-1}. The k_{cat} for p-nitroacetanilide was 5.8 s^{-1} while the K_m value of 0.06 mM was similar to published data (0.03 (Yoshioka et al., 1991); 0.07 (Hammond et al., 1983); 0.11 (Hwang and Chang, 1988)) and more than ten times lower than for hexanoamide. The capability to hydrolyse substituted amides of adipic acid was previously shown for an amidase signature enzyme from Achromobacter xylosoxidans with 5-[(5-carboxy-1-oxopentyl)amino]-2-nitro-benzoic acid where the enzyme had a K_m of 0.32 mM and a V_{max} of 150 IU mg^{-1} (Cai et al., 2005).

Various compounds were tested as potential inhibitors of the polyamidase using hexanoamide as substrate (Table III). The activity of polyamidase was completely inhibited with mercury chloride and silver nitrate while EDTA had no significant effect on the activity indicating that there was no metal ion in the catalytic center of the enzyme. This was in agreement to previous reports on aryl acylamidases (Hammond et al., 1983; Hirrlinger et al., 1996) and amidase signature enzymes (Toogood et al., 2004) which were also

Table III. Effect of different inhibiting compounds on the activity of the polyamidase from N. farcinica using hexanoamide as substrate.

Compound	IC$_{50}$ (mM)
Copper sulfate	3.2
Mercury chloride	0.2
Silver nitrate	0.2
1-Aminohexane	2.7
EDTA	—[a]
Calcium chloride	—
Cyanoacetamid	—
Manganese chloride	—
Zinc chloride	—
Ferrum sulfate	—
Magnesium sulfate	—

[a]No inhibition at 10 mM concentration (EDTA: 100 mM).

inhibited by heavy metals and showed no loss of activity with EDTA. In contrast to these data, the aryl acylamidase from N. globerula was not totally inhibited by mercury and silver and 1 mM EDTA effected an increase of activity (Yoshioka et al., 1991).

Homologies With Other Amidases

The 6-aminohexanoate-cyclic dimer hydrolase (EI) from Arthrobacter sp. KI72 had a similar subunit molecular weight of 52 kDa like the polyamidase but was a dimer (Negoro, 2000). Yoshioka et al. (1991) described a size of 126 kDa for the native aryl acylamidase from N. globerula and a size of 52 kDa for the subunit. Generally the molecular weights of native amidases and aryl amidases of other microorganisms varied from 35 to 480 kDa (Hirrlinger et al., 1996; Skouloubris et al., 1997).

The first 28 amino acids of the sequence of the polyamidase were identical to those of the aryl acylamidase from N. globerula (Yoshioka et al., 1991). The enzyme was similar with a putative amidase from N. farcinica IFM 10152 as indicated by 56% amino acid identity. The polyamidase belongs to the amidase signature family (Fig. 2) which comprises over 200 proteins from diverse origin including Bacteria, Archaea and Eukarya (Labahn et al., 2002). Unlike serine proteases, lipases and esterases which are characterized by the catalytic triad Ser-His-Asp, in the catalytic reaction of amidase signature enzymes the Ser-Ser-Lys triad is involved (Labahn et al., 2002; Valina et al., 2004). The corresponding catalytic mechanism has been recently confirmed based on the availability of the crystal structure of the Stenotrophomonas maltophilia peptide amidase (Pam). The strictly conserved amino acids Ser226, Ser202, and Lys123 of Pam forming the catalytic triad correspond to Ser173, Ser149, and Lys71 in the polyamidase of this study (Fig. 2).

Despite the common hydrolysis of amide bonds, individual representatives of the amidase signature family enzymes show very distinct substrate specificities (Valina et al., 2004). It has been suggested, that this is due to binding of the substrate by residues outside the signature sequence (Labahn et al., 2002). Nevertheless, within the amidase signature family an amidase cleaving cyclic nylon oligomers has been described (Negoro, 2000).

Linear and cyclic nylon oligomers are undesirable by-products in Nylon production which are released to the environment. Negoro and co-workers have described the enzymatic degradation of these by-products by bacteria extensively (Negoro et al., 2007). Several bacteria, including Arthrobacter sp. (formerly Flavobacterium sp.), were able to grow on the 6-aminohexanoate-cyclic dimer as the sole carbon and nitrogen source. Under these conditions 6-aminohexanoate hydrolases were produced. Three enzymes have been found to be involved in Nylon oligomer degradation by the Arthrobacter sp. KI72 and also by Pseudomonas sp. NK87 namely a 6-aminohexanoate-cyclic

Figure 2. Sequence alignment of the 6-aminohexanoate-cyclic dimer hydrolase (EI) from *Arthrobacter* sp. KI72 (formerly *Flavobacterium*; BAA05090), the peptide amidase (Pam) from *Stenotrophomonas maltophilia* (CAC93616), with a putative amidase from *Nocardia farcinica* (YP_119043). The catalytic triad characteristic for amidase signature enzymes is framed, highly conserved regions are marked in gray and peptide fragments of the polyamidase from *Nocardia farcinica* (this study) matching with the putative amidase from *Nocardia farcinica* (YP_119043) are underlined. GenBank accession numbers are given in brackets.

dimer hydrolase (EI), a 6-aminohexanoate-dimer hydrolase (EII) and an endo-type 6-aminohexanoate oligomer hydrolase (EIII) (Negoro et al., 2007). Apart from the cyclic and linear dimers, respectively, EI and EII did not hydrolyse more than 100 natural amino-compounds tested (Negoro, 2000). EIII hydrolyses the cyclic tetramer and dimer as well as linear oligomers endo-wise (Kakudo et al., 2000).

Interestingly, the 6-aminohexanoate-*cyclic* dimer hydrolases (EI) also belong to the amidase signature family like the polyamidase described in this study which are closely related (Fig. 3). In contrast, 6-aminohexanoate-*linear* dimer hydrolase activity (EII) has evolved in an esterase with β-lactamase folds. Consequently, unlike amidase signature family enzymes, the catalytic reaction of this enzyme is not characterized by the involvement of a Ser-Ser-Lys triad. Like for the penicillin-recognizing family of serine hydrolases, an

oxyanion of the substrate is formed as a common intermediate, which in case of the 6-aminohexanoate-dimer hydrolase is stabilized by the positively charged nitrogen at Ser112 and Ile345 (Negoro et al., 2007). Although one would expect closest similarity of the polyamidase from *N. farcinica* with the *endo*-acting 6-aminohexanoate oligomer hydrolase (EIII), this enzyme shows less homology than the *cyclic* dimer hydrolase (EI) and does not belong to the amidase signature family (Negoro et al., 2007).

Hydrolysis of Polyamide

Oxidases from lignolytic fungi have previously been shown to depolymerise PA (Deguchi et al., 1998; Friedrich et al., 2007; Klun et al., 2003). Peroxidases attack methylene groups adjacent to the nitrogen atoms and the reaction then
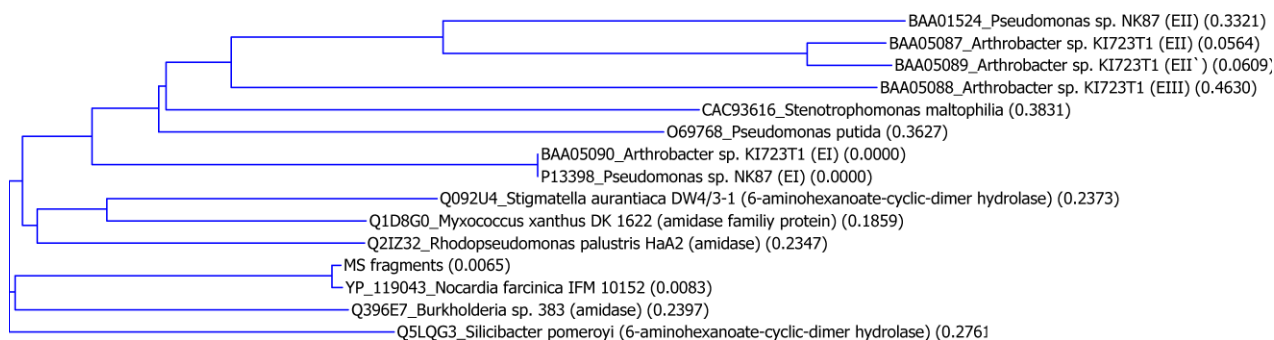

Figure 3. Phylogenetic tree based on amino acid sequences of the polyamidase from *Nocardia farcinica* (fragments as identified by MS) and other highly homologous bacterial amidases as well as amidases with substrate specificity for 6-aminohexanoate oligomers. The alignment was created using the Clustal W algorithm (Thompson et al., 1994). Tree calculation was based on a sequence distance method and utilized the Neighbor Joining (NJ) algorithm (Saitou and Nei, 1987). Accession numbers given refer to GenBank while *Arthrobacter* sp. was formerly named *Flavobacterium*. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

proceeds in an auto-oxidative manner (Deguchi et al., 1998; Nomura et al., 2001). However, oxidative enzymatic degradation of PA cannot be used for surface modification since the reaction is difficult to control leading to considerable damage of fibers. In contrast, an extracellular protease and a cutinase were successfully used for surface targeted hydrolysis of PA leading to hydrophilization (Miettinen-Oinonen et al., 2007; Silva et al., 2005). Here, we show for the first time that an intracellular amidase was also capable of hydrolyzing PA. Surface hydrolysis with this polyamidase lead to an increase of polar groups on the surface as measured with tensiometry (Table IV). The maximum increase is reached already after 3 min of incubation with no further changes over a period of 60 min of incubation.

Previous studies on enzymatic modification of polyethyleneterephthalate (PET) have demonstrated the existence of adsorbed protein (=enzyme) on the PET surface using angle resolved XPS. This led to misinterpretation of results on enzymatic surface hydrophilization (Vertommen et al., 2005). However, in previous studies on enzymatic hydrophilization of PA the possible contribution of protein to hydrophilization was not accounted for (Miettinen-Oinonen et al., 2007; Silva et al., 2005). In this study, the increase of hydrophilicity of PA-fabrics due to surface hydrolysis by the polyamidase was compared to mercury chloride inhibited controls. Thus, in contrast to previous studies it was clearly proven that enzymatic hydrolysis was responsible for hydrophilization while possible contribution of protein adsorption to hydrophilization was excluded (Fig. 4). After prolonged incubation the hydrophilicity reached a plateau and started to decrease. A similar behavior has been observed during enzymatic surface hydrolysis of PET and was attributed to the solubilization of smaller fragments from the surface (Almansa et al., in press).

Conclusion

An intracellular aryl acylamidase isolated from the soil bacterium *N. farcinica* was shown to hydrolyze polyamide fabrics thereby increasing the hydrophilicity. In contrast to previous studies, the contribution of protein adsorption to hydrophilicity increases was excluded by using enzyme inhibitors. Thus hydrophilicity increases can be clearly attributed to enzymatic surface hydrolysis. The polyamidase showed similar substrate specificities and was closely related to an aryl acylamidase from *N. globerula*. The polyamidase belongs to the amidase signature family like the
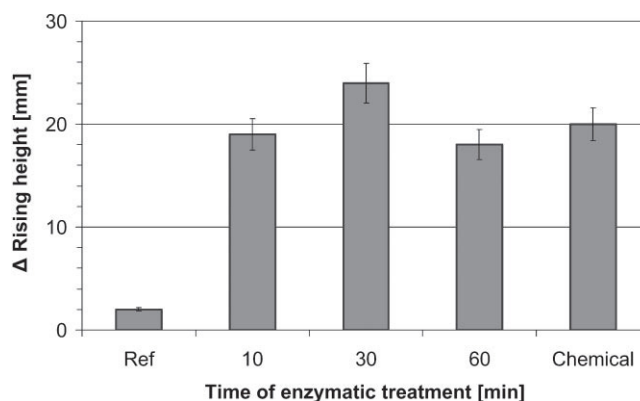

Figure 4. Increase of rising height of polyamide 6 fabrics after treatment with a polyamidase from *N. farcinica* compared to controls containing enzyme inhibitor and compared to the effect of silicone based finisher ("chemical"). Data represent the average of three measurements.

6-aminohexanoate hydrolase EI from *Arthrobacter* which cleaves (exclusively) cyclic dimers but not polyamides. In contrast, the 6-aminohexanoate hydrolase EIII from *Arthrobacter* hydrolyzing oligomers endo-wise was less closely related to the polyamidase *N. farcinica*. Nevertheless, this confirms previous assumptions that substrate specificities of amidase signature enzymes are strongly influenced by substrate binding by residues outside the signature sequence.

Apart from this polyamidase from *N. farcinica*, hydrolysis of polyamide has been reported for proteases and cutinases indicating that this ability is not characteristic to a certain class of enzymes. Future investigation should examine the power of genetic engineering to enhance the efficiency of polyamidases. Besides the adaptation of the architecture of the active site to fit polymeric substrates, the interaction of the enzyme and the substrate in terms of adsorption and desorption remains to be investigated and optimized.

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Table IV. Surface properties of polyamide 6 fabrics after treatment with a polyamidase from *N. farcinica* determined using tensiometry.

	t (min)	σ_{total} (mNm)	$\sigma_{dispers}$ (mNm)	σ_{spolar} (mNm)
Control	60	46.1	6.9	39.1
Polyamidase	3	63.7	5.4	58.2
Polyamidase	60	62.5	5.6	56.9

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