A Novel Aryl Acylamidase From \textit{Nocardia farcinica} Hydrolyses Polyamide

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\textbf{ABSTRACT:} An alkali stable polyamidase was isolated from a new strain of \textit{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 \textit{p}-nitrophenylbutyrate. On hexanoamide the amidase exhibited a \textit{K}_m value of 5.5 mM compared to 0.07 mM for \textit{p}-nitroacetanilide. The polyamidase belongs to the amidase signature family and is closely related to aryl acylamidases from different strains/species of \textit{Nocardia} and to the \textit{6-aminohexanoate-cyclic dimer hydrolase (EI)} from \textit{Arthrobacter} sp. K172.

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\textbf{KEYWORDS:} polyamide; amidase; \textit{Nocardia}

\section*{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 \textit{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 by 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 bis(hexylamide) 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 bis(hexylamide) (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\(_2\)PO\(_4\), 3.0 g L\(^{-1}\) K\(_2\)HPO\(_4\), 2.0 g L\(^{-1}\) NH\(_4\)Cl, 0.3 g L\(^{-1}\) MgSO\(_4\)\(\cdot\)7H\(_2\)O, 5 mL L\(^{-1}\) trace element solution with pH 7.0 and 0.8 g L\(^{-1}\) adipic acid bis(hexylamide) (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\(_2\)EDTA, 100 mg L\(^{-1}\) ZnSO\(_4\)\(\cdot\)7H\(_2\)O, 30 mg L\(^{-1}\) MnCl\(_2\)\(\cdot\)4H\(_2\)O, 300 mg L\(^{-1}\) H\(_2\)BO\(_3\), 200 mg L\(^{-1}\) CaCl\(_2\)\(\cdot\)6H\(_2\)O, 10 mg L\(^{-1}\) CuSO\(_4\)\(\cdot\)2H\(_2\)O, 900 mg L\(^{-1}\) Na\(_2\)MoO\(_4\)\(\cdot\)2H\(_2\)O, 30 mg L\(^{-1}\) Na\(_2\)SeO\(_3\)\(\cdot\)5H\(_2\)O, 1,000 mg L\(^{-1}\) FeSO\(_4\)\(\cdot\)7H\(_2\)O 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 33,000 g 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 μmol 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 μL) containing 200 μL potassium phosphate buffer (50 mM, pH 7.0), 20 μL of a 430 μM substrate solution and 100 μL enzyme solution or 100 μL buffer as a blank. The reaction was carried out at 30°C for 60 min and stopped with 32 μL 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 μL reaction mixture were added to 350 μL hypochlorite solution together with 350 μL 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 μL of the enzyme solution and 10 μL of the substrate solution (25 mg of p-nitroacetanilide dissolved in 1 mL DMSO and 30 μL Triton X 100). The increase of the absorbance at 405 nm which indicates an increase of p-nitroaniline due to hydrolysis of the substrate was measured at 25°C using a spectrophotometer type Hitachi U 2001. A blank was measured using 100 μL 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 of 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 of 50% water. A elution with 40% methanol, 10% 10 mM sulfuric acid and 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) and then centrifuged at 16,000g (Hereaus, Biofuge primo) and filtered with syringe filter (13 mm, with a temperature of 4°C). The enzyme purification was carried out 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\textsuperscript{®} bioseparation stirred cell (Millipore, Billerica, MA) was used with ultrafiltration membranes made of polyethersulfone 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 AKTA purifier 900. Enzyme activity was monitored using hexanoamide and adipic acid bishexylamide as substrates.

**Step 1:** Hydrophobic interaction chromatography: The intracellular fraction (10.5 mL) was equilibrated with 1 M Na\textsubscript{2}SO\textsubscript{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\textsuperscript{-1} flow) equilibrated with phosphate buffer (50 mM) and 1 M Na\textsubscript{2}SO\textsubscript{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\textsuperscript{-1} and the polyamidase was eluted at 60% elution puffer (1 M Na\textsubscript{2}SO\textsubscript{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 naphthyl 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-naphthyl)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 iodoacetamide 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 (Na₂HPO₄–2H₂O, 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 HgCl₂), 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
hexanoamide as substrate. This activity correlated with activity on the PA model substrate adipic acid bis(hexyl)amide 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.

### 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 amides.

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanoamide</td>
<td>100</td>
</tr>
<tr>
<td><em>p</em>-Nitrophenylbutyrate</td>
<td>669</td>
</tr>
<tr>
<td><em>p</em>-Nitroacetanilide</td>
<td>288</td>
</tr>
<tr>
<td>Butyramide</td>
<td>156</td>
</tr>
<tr>
<td>Methacrylamide</td>
<td>87.8</td>
</tr>
<tr>
<td>Propionamide</td>
<td>76.4</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>35.2</td>
</tr>
<tr>
<td>Acetamide</td>
<td>20.4</td>
</tr>
<tr>
<td>Asparagine</td>
<td>14.5</td>
</tr>
<tr>
<td>Mandelamide</td>
<td>7.6</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>1.2</td>
</tr>
<tr>
<td>Stearamide</td>
<td>1.1</td>
</tr>
<tr>
<td>Proteolytic activity with azocasein</td>
<td>0.00</td>
</tr>
<tr>
<td>Adipic acid bis(hexyl)amide</td>
<td>0.13</td>
</tr>
<tr>
<td>Bis (benzoyloxyethyl) terephthalate</td>
<td>0.04</td>
</tr>
</tbody>
</table>

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

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**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.]
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 (benzoyloxylethyl) 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 ary1 acylamidases from Rhodococcus sp. and Pseudomonas sp. (Hammond et al., 1983; Hirrlinger et al., 1996). Only the ary1 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 $k_{cat}/K_m$ for $p$-nitroacetonilide 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 xylosidans 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 ary1 acylamidases (Hammond et al., 1983; Hirrlinger et al., 1996) and amidase signature enzymes (Toogood et al., 2004) which were also inhibited by heavy metals and showed no loss of activity with EDTA. In contrast to these data, the ary1 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 ary1 acylamidase from N. globerula and a size of 52 kDa for the subunit. Generally the molecular weights of native amidases and ary1 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 ary1 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 byproducts in Nylon production which are released to the environment. Negoro and co-workers have described the enzymatic degradation of these byproducts 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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>$IC_{50}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper sulfate</td>
<td>3.2</td>
</tr>
<tr>
<td>Mercury chloride</td>
<td>0.2</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>0.2</td>
</tr>
<tr>
<td>1-Aminoctane</td>
<td>2.7</td>
</tr>
<tr>
<td>EDTA</td>
<td>—</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>—</td>
</tr>
<tr>
<td>Cyanocacatidem</td>
<td>—</td>
</tr>
<tr>
<td>Manganese chloride</td>
<td>—</td>
</tr>
<tr>
<td>Zinc chloride</td>
<td>—</td>
</tr>
<tr>
<td>Ferrum sulfate</td>
<td>—</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>—</td>
</tr>
</tbody>
</table>

*No inhibition at 10 mM concentration (EDTA: 100 mM).*
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 hydrolysates 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...
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 poly-ethyleneterephthalate (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 6-aminohexanoate hydrolase EI from *Arthrobacter* which cleaves (exclusively) cyclic dimers but not polyamides. In contrast, the 6-aminohexanoate hydrolase III 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.

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

<table>
<thead>
<tr>
<th></th>
<th>$\sigma_{\text{total}}$ (mN/m)</th>
<th>$\sigma_{\text{dispers}}$ (mN/m)</th>
<th>$\sigma_{\text{spolar}}$ (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>60</td>
<td>46.1</td>
<td>6.9</td>
</tr>
<tr>
<td>Polyamidase</td>
<td>3</td>
<td>63.7</td>
<td>5.6</td>
</tr>
<tr>
<td>Polyamidase</td>
<td>60</td>
<td>62.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>

6-aminohexanoate hydrolase EI from *Arthrobacter* which cleaves (exclusively) cyclic dimers but not polyamides. In contrast, the 6-aminohexanoate hydrolase III 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.

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**References**


Heumann et al.: A Polyamidase From Nocardia farcinica

Nocardia farcinica


