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ORIGINAL ARTICLE

Surface hydrolysis of polyamide with a new polyamidase from *Beauveria brongniartii*

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Abstract
Twelve fungi were screened for the potential of their extracellular enzymes to increase the hydrophilicity of polyamide (PA) materials. The most pronounced increase in hydrophilicity was found for enzymes from *Beauveria brongniartii* and *B. bassiana*. The 55 kDa polyamidase from *B. brongniartii* was purified using ultrafiltration, anion exchange chromatography and size exclusion chromatography. This polyamidase was able to hydrolyse adipic acid bishexylamide and various typical amidase substrates, but did not show protease activity. In contrast, the 27 kDa protease from *B. brongniartii* did not show activity on PA. The improvement of hydrophilicity due to hydrolysis with the 55 kDa polyamidase from *B. brongniartii* based on rising height was 11 cm for PA 6 Perlon fibres and 5 cm for PA 6.6 Nylon. The drop dissipation measurement corroborated the improvement of the hydrophilicity giving 7 s and less than 1 s for the two enzyme treated materials, respectively. The surface tension s of Perlon increased from 46.1 to 67.4 mNm after enzyme treatment.

Keywords: Beauveria brongniartii, *rising height, polyamide fibres, polyamidase*

Introduction

Limited surface hydrolysis of polyamides (PA) can increase hydrophilicity, which is essential for many applications including gluing, printing, electronics, biomedical, filtration and textile (Guebitz & Cavaco-Paulo 2008). For example, ultrafiltration membrane fouling by proteins increases the energy demand and requires cleaning with aggressive chemicals or even replacement. Higher hydrophilicity of ultrafiltration devices can enhance resistance to fouling (Reddy et al. 2005). Textile materials made of PA show poor water permeability due to their hydrophobicity. An increased hydrophilicity would enhance comfort to wear since sweat could penetrate and evaporate and static cling and stain retention during laundering would be reduced (Guebitz & Cavaco-Paulo 2008). The ability of a fibre to adsorb or to covalently bind dyes has, traditionally, been improved and enhanced by physical or chemical methods (Bendak 1991; Todorov & Valkov 1996; Makhlouf et al. 2007), which have recently been complemented by plasma and laser surface modification techniques (Wong et al. 2000; Ferrero 2003) or UV radiation methods (Lipp-Symonowicz & Sztajnowski 2003). However, most of the classic methods are, to some extent, environmentally harmful and/or rather complex when it comes to their technological application to the fibres (Battistel et al. 2001). Given these problems, enzymatic treatment of textiles could be an environmentally friendly, mild and, at the same time, a powerful alternative for improving the hydrophilicity of synthetic fibres.

Due to the chemical similarities of synthetic polyamides to their natural analogues the search for potentially active enzymes has focused on proteases (Silva et al. 2005; 2007). However, considering the large number of proteases available, very few have been found to be active on polyamides. Amidases like proteases, belong to the enzyme class of hydrolases, but they act on amide bonds, other

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than peptide bonds. In addition, cutinases have been shown to hydrolyse polyamides and genetic engineering has successfully achieved higher activity on synthetic polymers (Silva et al. 2006; Araujo et al. 2007).

Beauveria spp. are well known for the production of extracellular hydrolases including proteases (Agrawal et al. 2005; Erlacher et al. 2006). The objective of the present work was to isolate enzymes capable of modifying the surface properties of polyamide. This is the first report of a fungal polyamidase hydrolysing synthetic polyamides.

Materials and methods

Micro-organisms

Twelve fungi, namely Beauveria brongniartii, Trichoderma reesei bff120, Beauveria bassiana DSMC 344, Fusarium solani VAI 1, Botrytis cinerea, Aspergillus terreus A9, Bjerkandera adusta BAG1, Penicillium simplicissimum, Trichoderma viride BJG 102, Curvularia lunata IT 0005, Cylindrocarpon radicicola DSM 837 and Sclerotium rolfsii were assessed for their potential to produce extracellular enzyme hydrolysing PA.

Culture conditions

All fungi were grown on potato dextrose agar (PDA) plates (42 g l^{-1}) for 2 days at 30°C and afterwards stored at 4°C. To maintain the viability of the fungi, they were replated every 3 months. The medium for liquid cultivation contained yeast extract (1.5 g l^{-1}), glucose (1.0 g l^{-1}), $MgSO_4.7H_2O$ (4.0 g l^{-1}), $(NH_4)_2SO_4$ (1.0 g l^{-1}), KH_2PO_4 (2.5 g l^{-1}) and Na_2HPO_4 (2.5 g l^{-1}) in tap water. The pH was adjusted to 6.5 using HCl. Wheat bran (10.0 g l^{-1}) was added as additional carbon source. One-litre Erlenmeyer flasks filled with 400 ml medium were inoculated with 1 vol% from the fungal plates and incubated on a rotary shaker at 30°C and 130 rpm for 5 days except for *S. rolfsii*, which was grown for 10 days.

Protein concentration

Protein concentration was determined following Bradford's method using a Bio-rad kit. 100 l enzyme solutions were added to 700 l 25 mM phosphate buffer (pH 6.0) and 200 l Bio-Rad reagent and the change in absorption at 595 nm was recorded using a Hitachi U-2001 spectrophotometer. The method was calibrated using bovine serum albumin (BSA) as reference.

Enzyme activity

Esterase activity was determined by monitoring the hydrolysis of *p*-nitrophenyl-propionate as substrate photometrically at 405 nm at 25°C. One unit was defined as the amount of enzyme cleaving 1 μmol of ester min^{-1} under the given assay conditions.

Protease activity was determined according to a method previously published (Tomarelli & Charney 1949) using azocasein. Two per cent azocasein was dissolved in 25 mM phosphate buffer at pH 7.0 and clarified by centrifugation at 12,000 ***g*** for 10 min. A 250-l sample of the azocasein solution were added to 150 l enzyme solution and incubated at 37°C for exactly 30 min. The reaction was stopped with 1.2 ml of 10% w/v trichloro-acetic acid, then centrifuged at 8000 ***g*** for 5 min. The supernatant (1.2 ml) was mixed with 1.4 ml 1 M NaOH and the resulting change in absorption monitored at 440 nm. One unit was defined as the amount of enzyme necessary to produce an absorbance change of 1.0 (in a 1-cm cuvette) per minute, under the conditions of the assay.

Amidase activity was determined by measuring the amount of NH_3 liberated during the hydrolysis of nine different substrates (Cramp & Gilmour 1997), namely acetamide, propionamide, butyramide, benzamide, nicotinamide, mandelamide, methyl-acrylamide, cyanacetamide, asparagine, hexanamide and stereamide. Twenty microlitres of substrate solution were added to 100 l enzyme solution and 200 l bis-tris buffer (pH 6.0), and subsequently incubated for 30 min at 50°C. One-hundred microlitres of the reaction mixture were added to 350 l of hypochlorite reagent (0.11 M hypochlorite and 2 M NaOH). Subsequently, 350 l of Na-nitroprusside reagent (0.59 M phenol and 0.01 M Na-nitroprusside) were added. After incubation at 50°C for 15 min the resulting indophenol was monitored photometrically at 635 nm. One unit was defined as the release of 1 μmol of NH_3 min^{-1}.

Enzyme purification

For ultrafiltration an Amicon ultrafiltration cell (Amicon, Beverly, Ma, USA) with PES membranes (30 kDa) was used. Filtration was performed under ice-cold conditions and a pressure (pressurized air) of 3 bar using a sample volume of 1000 ml. Cleaning of the membranes was performed with 0.1 M NaOH solution. Both concentrated phase and filtrates were stored at −20°C for further use. For chromatographic purification of the enzyme an ÄKTA explorer system with a PC-based UNICORN control system (version 3.2) and a Frac-900 collector was used. In the first step, an anion exchange (DEAE) HiTrap Q4 column was used as stationary phase and

20 mM bis-tris buffer (pH 6.0) as mobile phase (flow rate 5 ml min^{-1}). Samples of 4.5 ml were applied to the column and eluted in the same buffer with a 1 M NaCl using a 15 −35–100% gradient. For subsequent size exclusion chromatography, the system was equipped with a Superdex 75 HR 10/30 column. To eliminate any unwanted electrostatic biasing in the separation process the 20 mM bis-tris buffer contained 0.1 M in NaCl. 0.2-ml samples was applied and eluted at 0.5 ml min^{-1}. The fractions (0.5 ml) showing enzyme activity were concentrated and desalted using a Vivaspin 20-unit ultrafiltration apparatus (Vivascience, Sartorious AG) supplied with 5 kDa MWCO PES membranes. The obtained samples were centrifuged at 7000 ***g*** for 30 minutes. The resulting concentrated and desalted samples were stored at −20°C.

Gel electrophoresis

Gel electrophoresis was performed with a Mini-PROTEAN 3 Electrophoresis System (Bio-Rad). The 12% SDS-PA gels were run with the unknown samples and pre-stained molecular weight markers covering a broad range of molecular weights (200–14.4 kDa) applying 180 V (DC). The gel was stained using Coomassie Blue R-250 for 30 min, and afterwards destained with 40% ethanol and 10% acetic acid.

Temperature, pH-optima and inhibition

The temperature (at pH 6.5) and pH dependence of the (purified) enzyme activity was tested using the amidase activity assay with propionamide as substrate (incubation time 30 min) and by monitoring the decomposition of the PA 6.6 model-substrate (incubation times 24 and 48 h). For determination of the pH optimum (at 50°C) 20 mM citric acid buffer ($2.5 < pH < 6.0$) and 20 mM Tris-buffer ($pH > 6.0$) were used.

The inhibitory effect of $CuSO_4$, $HgCl_2$, $AgNO_3$, hexylamine, $CaCl_2$, $MnCl_2$, $ZnCl_2$ and $FeSO_4$ was studied. 100 l enzyme preparations were incubated with 500 l of 5 mM inhibitor solution for 30 min at 25°C, then the amidase activities were evaluated using the amidase activity assay with propionamide as substrate.

Enzyme hydrolysis of polyamide fibres

Nylon (PA 6.6, 120 g m^{-2}) and Perlon (PA 6, 250 g m^{-2}) fabrics were supplied by CIBA and Multifil fabrics (PA 6.6, 470 g m^{-2}) were from RHODIA. Prior to the enzymatic treatment the fabrics were washed with Na_2CO_3 (1 g l^{-1}; pH 10) for 30 min and subsequently immersed in water for 10 min to remove the surface finishes. Pieces of 2 × 20 cm were treated in 50-ml enzymatic solutions (extracellular enzymes from the 12 fungi in their respective buffer solutions) in 250-ml flasks on a shaker at 120 rpm and 30°C for 30 min, 1 and 5 h. Control samples with heat denatured enzymes, added inhibitors or without any enzyme, were prepared and treated accordingly. After the treatment, the fibres were washed with 1 g l^{-1} Na_2CO_3 (pH 10) solution for 2 hours, rinsed with distilled water (4 times) to remove residual enzyme or other media impurities and the fabrics dried at room temperature overnight.

Measurement of surface hydrophilicity. A slightly modified method from DIN 53924 was applied for the measurement of rising height. It consisted of hanging the fabrics (cm) on a glass rod and suspending the lower part in a bath of distilled water. Afterwards

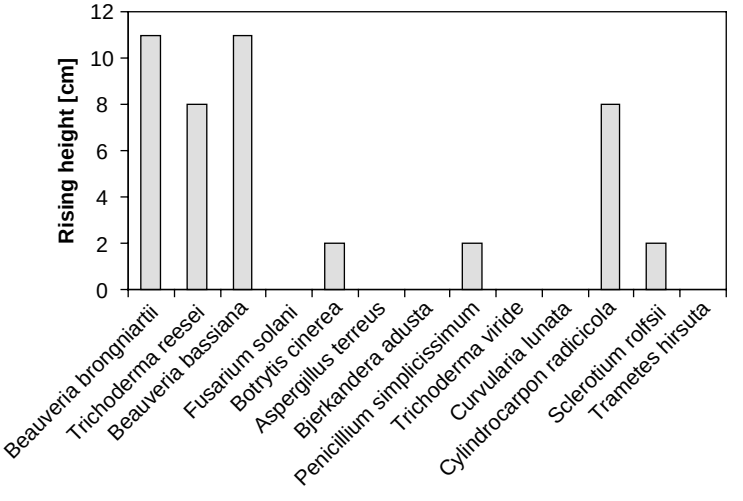

Figure 1. Rising height of PA 6 Perlon fibres after treatment with enzyme preparations from 12 different fungi.

the level of the adsorbed water on the fabric was measured.

The drop dissipation measurements were carried out as described by Li & Hardin (1998) and applied to PA by (Heumann et al. 2006). A drop of distilled water (20 l) was placed on the surface of the fabric. The time for the fabric to take up the drop was measured. All measurements were carried out in three replicates. For surface tensiometry the K 100 apparatus from Krüss (Hamburg, Germany) was used. The surface tension s in mNm of the samples was determined in the Wilhelmy-type automatic surface balance mode at 25°C.

Enzyme hydrolysis of the PA 6.6 model substrate

Adipic acid bishexylamide was synthesized and purified as previously described (Heumann et al. 2006). For analysis of the hydrolysis products a gas chromatograph (5890 HP Split/Splitless-Injector and FID-Detector, using INNOWAX column) was used. One-hundred microlitres of the filtered sample were transferred to the GC vial and separated with an INNOWAX column at a flow rate of 1.85 ml min^{-1} at 280°C. The apparatus was calibrated using hexylamine standard solutions in the concentration range from 0.1 to 10 mM at a neutral pH. The quantification of adipic acid was made by HPLC using a Discovery Hs 15 cm C18 from Supelco with a mobile phase of 10% H_2SO_4, 8% acetonitrile and 82% water. The flow rate was 1 ml min^{-1} at 40°C with detection at a wavelength of 220 nm.

Results and discussion

Screening for polyamidases

The potential of extracellular enzyme preparations from 12 fungi for limited surface hydrolysis of polyamides was assessed. Newly-generated carboxylic acid groups on the surface should increase the hydrophilicity of polyamide materials, which would be highly desirable for application as textiles or ultrafiltration devices (Guebitz & Cavaco-Paulo 2008; Reddy et al. 2005). In addition to the rising height technique for quantification of hydrophilicity increases, hydrolysis products (i.e. adipic acid and hexylamide) of a PA 6 a model substrate were quantified using HPLC and GC as previously described (Heumann et al. 2006).

In the screen, six of the 12 tested fungi, namely *F. solani*, *A. terreus*, *B. adusta*, *T. viride*, *T. hirsuta* and *C. lunata* did not give positive results, with their rising heights being equal to the initial reference of PA 6 (i.e. 0 cm). While *T. reesei* and *C. radicicola* showed a positive effect on the rising height of the polymer (Figure 1), the best results were obtained with *Beauveria bassiana* and *B. brongniartii*, which are both known for the production of proteases (Leopold 1970; Leger & Cooper 1986; Shimizu & Tsuchitani 1993; Chen et al. 2000; Erlacher et al. 2006). Due to the availability of a purified protease from *B. brongniartii* in our laboratory (Erlacher et al. 2006), this species was chosen for further investigation. Interestingly, this purified 27 kDa protease did not show any activity on PA or on the PA model substrate. In agreement with this observation, protease activity did not correlate with rising height increases when extracellular enzyme samples taken at different cultivation times of *B. brongniartii* were applied (Figure 2). In contrast to these results, increases in hydrophilicity of polyamides have previously been achieved after treatment with a protease from *B. subtilis*. The increased hydrophilicity also led to enhanced binding of reactive dyes. During enzyme treatment, polyamide hydrolysis products were detected in the incubation mixture (Silva et al. 2006).

The active enzyme preparation from *B. brongniartii* hydrolysed the model substrate adipic acid

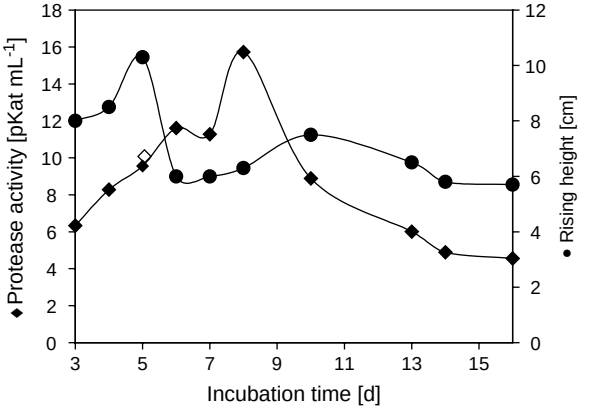

Figure 2. Effect of the cultivation time of *B. brongniartii* on protease activity and on the rising height of PA6 Perlon fibres after incubation with extracellular enzymes.

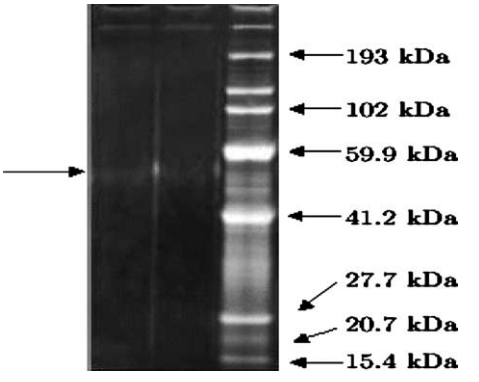

Figure 3. Negative image of the SDS gel of the purified polyamidase from *Beauveria brongniartii* (left two lanes) and molecular weight markers (right lane).

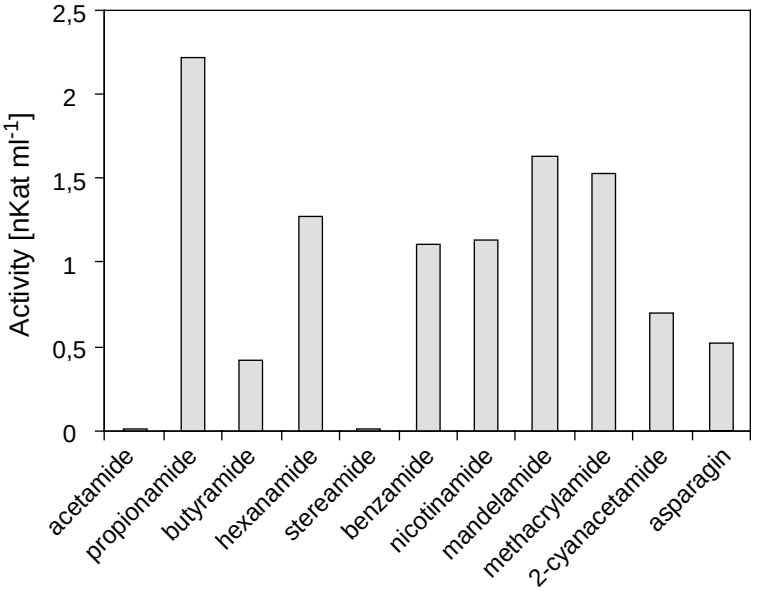

Figure 4. Activity of the *B. brongniartii* polyamidase on different substrates.

bishexylamide based on detection of hexylamine with an activity of 61 nkat mg^{-1} (purified protein). However, adipic acid was never detected indicating that the polyamidase cannot hydrolyse both bonds in the model substrate. Therefore, the release of hexylamine from the model substrate was used for the determination of polyamidase activity during enzyme purification since a correlation to activity on PA has been described previously (Heumann et al. 2006). In contrast, enzymes hydrolysing other PA oligomers (i.e. 6-aminohexanoate dimer) were not capable of hydrolysing amide bonds in polymeric substrates (Negoro 2000).

Enzyme purification and characterization

The polyamidase from *B. brongniartii* was purified using ultrafiltration (30 kDa MWCO), anion exchange chromatography and size exclusion chromatography. After separation by anion exchange chromatography, the polyamidase active fraction (highest activity on model substrate and on methylacrylamide) did not show any protease activity; however, esterase activity was detected. According to SDS-PAGE the enzyme had a molecular mass of 55 kDa (Figure 3). The enzyme showed amidase activity with both aliphatic and aromatic substrates

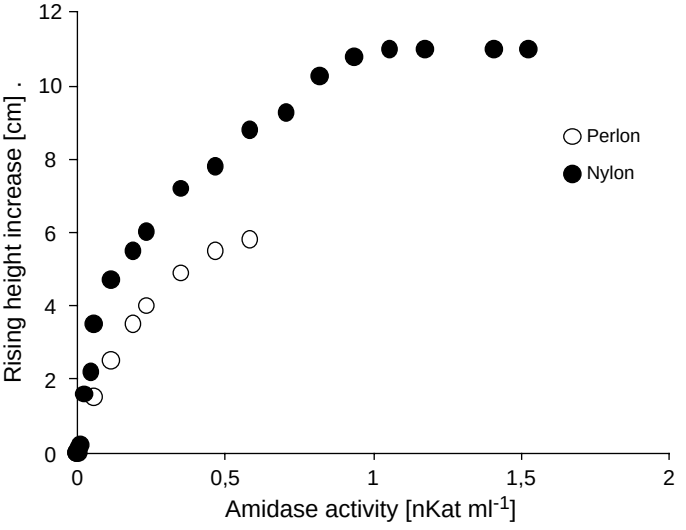

Figure 5. Increase of rising height of PA 6 Perlon and PA 6.6 Nylon after treatment with the amidase from *B. brongniartii* at 30°C and pH 6.5 for 60 min.

(Figure 4). Compared with longer chain amides up to C6 the activity on acetamide was low. The pH optimum of the polyamidase was 6 for both propionamide and the model substrate. The temperature optimum was 60°C.

Several amidase inhibitors were tested showing that $AgNO_3$ (92% inhibition), $HgCl_2$ (100% inhibition) and cyanacetamide (100% inhibition) strongly inhibited the polyamidase, while moderate inhibition was seen with $MnCl_2$ (54% inhibition).

Treatment of polyamide fabrics

The *B. brongniartii* polyamidase was able to increase the hydrophilicity of both PA 6 (Perlon) and PA 6.6 Nylon and Multifil.

The dependence of the rising height of PA 6 Perlon and PA 6.6 Nylon on the amidase activity from the extracellular enzymes from *B. brongniartii* is shown in the Figure 5. The initial rising height for Perlon and Nylon were 0 and 2.5 cm, respectively. The drop dissipation measurement confirms the results obtained for rising height. For both substrates, a rapid initial decrease with increasing amidase activity was seen which levelled off to a plateau value once a certain amidase activity was reached. For PA6 the initial value of 60 s was reduced to 7 s at an amidase activity of 0.1 nkat ml^{-1} and 60 min treatment. In addition to Perlon and Nylon a PA 6.6 fabric Multifil from Rhodia was tested. This material was already relatively hydrophilic with a rising height of 5 cm. Nevertheless, polyamidase treatment led to a further increase of the hydrophilicity to 8 cm in terms of rising height. Apart from the catalytic action of the enzyme, protein adsorption may also contribute to hydrophilicity increases (Guebitz & Cavaco-Paulo 2008). However, neither heat-inactivated polyamidase nor bovine serum albumin at the same protein concentration as the polyamidase produced any increase in rising height.

The effect of surface hydrolysis on surface tension of PA 6 Perlon fabrics was measured using tensiometry. As expected, the surface tension s increased after 3 min enzyme treatment from 46.1 to 67.4 mNm. However, after 60 min incubation this effect was reduced to 63.8 mNm and to the level of the blank (45.7 mNm) after 120 min. We speculate that prolonged incubation might lead to extensive surface hydrolysis causing solubilization of the upper layer (i.e. oligomers) of the material thus reducing again the number of new carboxylic acid groups on the surface.

In conclusion, we report a novel polyamidase from *B. brongniartii* which can hydrolyse polyamides, a PA oligomer and various amidase substrates, but which does not show protease activity. Future investigations should focus on structural requirements of enzymes for activity on polyamides. Such studies should include cutinases, proteases, and amidases, where few representatives have previously been shown to hydrolyse polyamides.

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