Characterization of a New Extracellular Hydrolase from *Thermobifida fusca* Degrading Aliphatic−Aromatic Copolyesters

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The paper describes the purification, biochemical characterization, sequence determination, and classification of a novel thermophilic hydrolase from *Thermobifida fusca* (TfH) which is highly active in hydrolyzing aliphatic−aromatic copolyesters. The secretion of the extracellular enzyme is induced by the presence of aliphatic−aromatic copolyesters but also by adding several other esters to the medium. The hydrophobic enzyme could be purified applying a combination of $(NH_4)SO_4$-precipitation, cation-exchange chromatography, and hydrophobic interaction chromatography. The 28 kDa enzyme exhibits a temperature maximum of activity between 65 and 70 °C and a pH maximum between pH 6 and 7 depending on the ion strength of the solution. According to the amino sequence determination, the enzyme consists of 261 amino acids and was classified as a serine hydrolase showing high sequence similarity to a triacylglycerol lipase from *Streptomyces albus* G and triacylglycerol-aclyhydrolase from *Streptomyces sp.* M11. The comparison with other lipases and esterases revealed the TfH exhibits a catalytic behavior between a lipase and an esterase. Such enzymes often are named as cutinases. However, the results obtained here show, that classifying enzymes as cutinases seems to be generally questionable.

Introduction

Plastics that are controlled degradable in a biologically active environment have been developed during the last 15 years. The main driving force for these developments was to open composting as an alternative waste treatment system for plastics and to add biodegradability as a novel material property to plastics for special applications (e.g., degradable mulching films in agriculture). Although in the first phase material development and a general evaluation of the biodegradability (based on standardized tests) were given emphasis, nowadays increasing interest is focused on the detailed investigation of the mechanisms of the microbial degradation process of these macromolecular materials.

Most of the polymeric materials used as biodegradable plastics are insoluble in water and, thus, in principle not bioavailable. To use natural polymers (e.g., cellulose, starch, lignin, proteins, etc.) or poorly water soluble natural substances such as oils and fats as feedstock for microorganisms, nature developed a number of special extracellular enzymes increasing the bioavailability of such substances by means of degradation processes of the polymeric material. The enzymes are secreted into the environment by the microbial cells, attach somehow to the phase interface and cleave the substrate there as long as water-soluble small intermediates are formed which can be transported into the cells and metabolized by the intracellular biochemical pathways. The same mechanism as for natural polymers is anticipated for biodegradable plastics, either if they originate from natural polymers or from chemical feedstock.

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The group of biodegradable plastics with commercial relevance is dominated by polyesters or polyester-based materials. One of the first biodegradable plastics on the market was the natural polyester poly(*β*-hydroxy butyrate-*co*-*β*-hydroxy valerate) (PHBV) which is produced and intracellularly accumulated by a number of bacteria.[1,2] However, due to a better cost performance and improved use properties, synthetic polyesters were predominantly applied as biodegradable plastics. A number of aliphatic polyesters proved to be susceptible to a microbial attack at a rate suitable for use as controlled biodegradable materials. Unfortunately, most of the aliphatic polyesters lack in satisfactory material properties (especially the thermal behavior due to low melting points), and thus, attempts were undertaken to improve the desired properties of these materials. Since pure aromatic polyesters, including poly(ethylene terephthalate) (PET) or poly(butylene terephthalate) (PBT), are microbially resistant, the developments focused on aliphatic−aromatic copolyester, and a variety of such polyesters are produced now on a commercial scale.[3]

Although for PHB, PHBV, and other polyhydroxyalkanoates a special group of hydrolytic enzymes, called PHB-depolymerases,[4,5] is responsible for the extracellular depolymerization, it turned out that synthetic polyesters are predominantly attacked by lipases[6] or enzymes classified as cutinases.[7] Recently, polymer-related factors controlling the enzymatic catalysis of polyester hydrolysis by a lipase from *Pseudomonas sp.* were investigated systematically by Marten et al.[8,9] and Welzel.[10] For the majority of investigations on the enzymatic hydrolysis of polyesters, commercially available enzymes were applied.

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Recently, various thermophilic actinomycetes were isolated by Kleeberg et al.[11,12] and isolates belonging to the species of *Thermobifida fusca* turned out to be highly active in degrading aliphatic−aromatic copolyesters. The corresponding degrading extracellular enzyme was isolated as well and was characterized with regard to its production. In the present paper, purification, biochemical properties as well as the amino acid sequence of this enzyme are presented and the results are discussed in comparison with other hydrolases.

Materials and Methods

Microorganism. The enzyme was obtained from the actinomycete *Thermobifida fusca (former name: Thermomonospora fusca*) DSM 43793. The strain was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The thermophilic actinomycetes exhibit a maximum growth rate at 50−55 °C and a neutral pH.

Polymers. The aliphatic−aromatic copolyester BTA40:60 (M_w = 47 600 g/mol), synthesized by melt polycondensation from 1,4-**b**utanediol, dimethyl **t**erephthalate, and **a**dipic (abbreviation "BTA" polymers), was obtained from the Hüls AG (Marl, Germany). BTA45:55 (M_w = 66 000 g/mol) was kindly supplied by BASF AG (Ludwigshafen, Germany). The molar ratios of adipate and terephthalate monomers were 40:60 for BTA40:60 and 45:55 for BTA45:55. Poly(ϵ-caprolactone) (Tone 787) from Union Carbide (Union Carbide, Danbury CT) was used for an accelerated activity test of the enzyme.

Polybutyleneadipate (SP4/6) and aromatic oligo-esters of (BT and BTBTB with B = 1,4-butanediol-unit and T = terephthalate unit) were synthesized by melt condensation from 1,4-butanediol adipic acid and dimethylene terephthalate, respectively, using zinc acetate dihydrate as catalyst and purified by an extraction and precipitation procedure with methanol and water. Details are described elsewhere.[8]

Polymer films of defined thickness were prepared by a melt-pressing technique applying a thermostated press (Perkin-Elmer, Überlingen, Germany). The temperature during pressing was chosen 5 °C below the melting point of the materials, a force of two tons was applied for 2 min to prepare the films.

Polyester nanoparticles were prepared by a special precipitation method described by Gouda et al.[13]

Enzymes. Lipases from *Pseudomonas sp.* (PsL) was purchased from Sigma, German and the Lipase from *Aspergillus oryzae* (AoL), the lipase from *Candida cylindracea* (CcL) and the esterase from *Bacillus stearothermophilus* was purchased from Fluka, Germany.

Chemicals. Dibutyl terephthalate was synthesized from dimethyl terephthalate and butanediol with tetraisopropylorthotitanate as catalyst as described by Marten.[8] Dimethyl terephthalate (Fluka), dibutyl phthalate (Merck), and dimethyl phthalate (Merck) were used with a purity higher than 99%.

Cutin was prepared from apple skins according to a method described by Walton et al.[14]

Cultivation of *Thermobifida fusca* DSM 43793. The TSB medium (tryptic soy broth) and MSV medium (mineral salt medium + vitamins) used for the cultivation of *T. fusca* is described elsewhere.[13] As a pre-culture, 200 μL of a spore suspension (approximately 10^7 spores/mL) was incubated in 5 mL of TSB medium in culture tubes for 18−24 h at 55 °C on a rotary shaker (120 rpm). Pellets of the actinomycete from this pre-culture were then homogenized with an Ultraturrax (8000 rpm, 15 s).

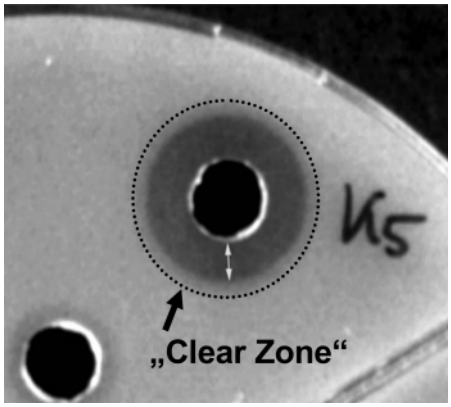

Figure 1. Clear zone formation on a agar plate containing fine dispersed BTA45:55 copolyester.

Erlenmeyer flasks filled with medium up to a height of 2 cm were inoculated with 1 vol % of pre-culture and incubated on a rotary shaker at 55 °C and 120 rpm for 18 h.

Tests for Enzyme Activity. *Induction Experiments.* The activity was determined from the acids released from PCL films during the enzymatic hydrolysis of the ester bonds. The procedure is described in detail by Gouda et al.[13] One activity unit is defined as the amount of enzyme that cleaves 1 μmol esters per minute.

Clear Zone Test. Monitoring active fractions during the chromatographic purification was performed using a semi-quantitative "clear zone" test. The occurrence of a clear halo around a drop of enzyme solution on an opaque agar which contains finely dispersed BTA copolyester indicates a polyester degrading activity (Figure 1).

Turbid agar plates were prepared by mixing agar solution (20 g/L agar in 80 mM phosphate buffer pH 7.1) with a suspensions of the BTA45:55 copolyester (30 g/l) finally obtaining an agar concentration of 1.5 g/L. The solution was autoclaved (121 °C, 20 min) and after cooling to approximately 50 °C poured in Petri dishes (4 mL of agar solution each). The enzyme solution (30 μL) was given in small holes punched out of the agar. The plates were incubated at 55 °C for 15 h.

UV Test. pH and temperature optimum of the purified enzyme was characterized by a test determining the release of free terephthalic acid in a buffer by UV detection (242 nm). To 2.95 mL of buffer and 500 μL of the enzyme solution (500 μg/mL protein) in a quartz cuvette were added four circular films (ø 5 mm) of the copolyester BTA 45:55. The increase in UV absorption was monitored with a Lambda 15 UV-photometer (Perkin-Elmer, Überlingen, Germany). The cuvettes were thermostated with a water bath and were shaken every 15 min to agitate the films in the solution. Activity was expressed as increase in extension with time.

Titration Assay. For a better quantitative determination of enzyme kinetics, the activity was monitored via the release

of free acids during ester cleavage with a auto-titration system in sterile NaCl solution (9 g/L) at pH 7. A total of 6 mL of NaCl solution with the enzyme sample was given into a 10 mL thermostated glass reactor. The reaction was started by the addition of two sterile polyester films (ø 0.9 cm) or a nanoparticle suspension containing 0.7 mg nanoparticles. The ester cleavage rate was calculated from the consumption of 0.1 M NaOH necessary to keep the pH at 7.

Liquid substrates (triglycerides) were tested in a special emulsifying solution (4.475 g of NaCl, 0.103 g of KH_2PO_4, 135 mL of glycerol, 75 mL of water, and 1.5 g of Gummi Arabicum). The triglyceride (0.5 mL) was mixed with 5 mL of the emulsifying solution and 4.5 mL of water and treated for 1 min with an Ultraturrax at 13500 rpm. This emulsion (6 mL) was then filled into the titration chamber, and the pH was adjusted to 7.1 ± 0.1 and thermostated to 55 °C. The test was started by addition of 20 μg of the enzyme.

Enzyme Purification. *Ammonium Sulfate Precipitation*. To precipitate the proteins of the culture broth $(NH_4)_2SO_4$ was slowly added in small portions to the broth under stirring at 4 °C. An ammonium sulfate concentration of 50% of the saturation level proved to precipitate the complete BTA-hydrolyzing activity from the solution. To complete precipitation the broth was stirred for another 2 h and then the precipitate was centrifuged (at a centrifugal force of 44 000 g for 30 min at 4 °C). The enzyme was redissolved in the buffer according to the following purification step.

Ultrafiltration. For ultrafiltration an Amicon model 8050 ultrafiltration cell (Amicon, Beverly, Ma, USA) with regenerated cellulose membranes (47 mm, UF -membrane RC, Membrapur GmBH, Bodenheim, Germany) was used. Filtration was performed at 4 °C and a pressure (pressurized air) of 3 bar. Cleaning of the membranes was done with 0.1 M NaOH solution.

Chromatography. For chromatographic purification of the enzyme a LCC-500 Plus FPLC-system (Pharmacia, Uppsala, Sweden) was used. The columns were purged with a 20-fold column volume of the start buffer before sample application (sample solutions filtrated through 0.2 μm Minisart NML filters, Sartorius, Göttingen, Germany). Composition of the buffers (degassed and filtered through 0.2 μm regenerated cellulose membranes) and flow rates are given with the corresponding results. The following ready made columns and gels, respectively, were used: Q-Sepharose Fast Flow (Q 1126, Sigma, Deisenhofen, Germany); S−Sepharose Fast Flow (85636, Fluka, Deisenhofen, Germany); Uno-S1 (720−0021, BioRad, Munich, Germany); Sepharose 4B (84962), Fluka, Deisenhofen, Germany); Phenylsepharose CL-4B (17−0810−01, Pharmacia Biotech, Freiburg, Germany); HiTrap HIC Test Kit (17−1349−01,Pharmacia Biotech, Freiburg, Germany). The chromatographic purification was performed at room temperature. Fractions with BTA-degrading activity were identified with the clear zone method (see above).

Gel Electrophoresis. For analyzing the purification effect of the chromatographic methods, analytical gel electrophoresis was performed with a Multiphor II Gelelectrophoresis-System (Pharmacia Biotech., Uppsala, Sweden) applying Excel-Gels (SDS Gradient 8-18, Pharmacia Biotech., Uppsala, Sweden) with a 15 μL sample volume at 15 °C and 600 V/50 mA for 75 min. Bands were silver stained.

To provide pure material for the sequence determination of the enzyme, a Mini-Protean-II−Gel-System (Biorad, Munich, Germany) was used (gels 7 × 10 cm, 1 mm thickness).

The stacking gel (5.1%) consisted of 3.2 mL of H_2O, 1.25 mL of Tris (0.5 M, pH 6.8), 0.5 mL of acrylamide (300 g/L)/ *N*,*N*-methylenebisacrylamide (8 g/L), 50 μL of SDS (100 g/L), 15 μL of TEMED (*N*,*N*,*N*′,-tetramethylethylenediamine), and 60 μL of APS (ammoniumpersulfate, 100 g/l). Separation gel consisted of 3.6 mL of H_2O, 3.8 mL of Tris (1.5 M, pH 8.8), 7.5 mL of acrylamide (300 g/L)/ *N*,*N*-methylenebisacrylamide (8 g/L), 150 μL of SDS (sodium dodecyl sulfate, 100 g/L), 20 μL of TEMED, and 120 μL of APS (100 g/L).

The stacking buffer was composed as follows: 62.5 mM Tris pH 6.8, 30 g/L glycerol, 60 g/L SDS, 15% (v/v) β-mercaptoethanol, 0.25 g/L bromophenol blue. Separation buffer: 25 mM Tris pH 8.6, 192 mM glycin, 1 g/L SDS.

A sample volume of 40 μL at a protein concentration of 200 μg/mL was applied. Separation was performed at 80V/120V until the separation buffer reached the end of the gel. Bands were Coomassi Blue stained.

Determination of the Amino Acid Sequence. For the determination of the amino acid sequence, two aliquots of the enzyme preparation were separated by SDS gel electrophoresis and transferred to a PVDF membrane (polyvinylidene fluoride, Immobilon-PS Q Transfer Membran/ Millipore, Eschborn, Germany) by Western Blotting (Mini Transblot Electrophoretic Transfer Cell/Biorad, Richmond, USA). After Coomassi Blue staining of one membrane, the corresponding enzyme spot on the second, unstained membrane was cut off and the protein was digested with trypsin and GluC. The separation of the peptides was then performed by reversed phase HPLC. A 473A-Sequencer (Applied Biosystems, Foster City, USA) was used to analyze the peptide sequences, and the total sequence was obtained by overlapping the fragments and by comparison with two lipases of streptomyces.

Results and Discussion

Induction of the BTA hydrolase. In preliminary investigations, it turned out that the hydrolysis of BTA40:60 films (weight loss) with cultures of *T. fusca* DSM 43793 in a mineral salt medium was increased significantly by the addition of pectin, peptone, and trypic soy broth. In contrast, by using media supplemented with saccharides and polysaccharides (glucose, mannose, xylan, xanthan, cellulose, and starch), no increased degradation by *T. fusca* was observed compared to the blank test (mineral medium without supplements).

It could be supposed that pectin and peptone induce the production of the extracellular, BTA degrading enzyme (BTA hydrolase) since most of the extracellular substrate degrading enzymes are known to be inducible.[15] However, when growing *T. fusca* without the presence of the BTA polymer, no enzymatic activity could be detected in the culture

Table 1. Induction of BTA hydrolase Activity during the Culture of *Thermobifida Fusca* DSM 43793 by Addition of Various Ester in Different Forms[a]

substance used for induction	activity in the culture medium (U/mL)[b]
BTA 40:60 films	0.64
BTA 45:55 powder	0.50
BTA 45:55 nanoparticles[c]	0.10
BT[d]	0.24
BTBTB[e]	0.65
dibutylterephthalate	0.26
dimethylterephthalate	0
dibutylphthalate	0
dimethylphthalate	0
poly(ϵ-caprolactone)	0.38

[a] Culture in MSV-medium at 55 °C for 48 h; medium volume 300 mL; addition of 25 mg ester. [b] One unit is defined as the amount of enzyme which cleaves 1 μmol esters of PCL films per minute in a test described by Gouda et al.[13] [c] Only 6 mg BTA used for induction instead of 25 mg. [d] BT is the monoester of terephthalic (T) acid and 1,4-butanediol (B); the synthesis is described by Marten.[8] [e] BTBTB is the oligoester of two terephthalic acid units (T) and three molecules of 1,4-butanediol (B); the synthesis is described by Marten.[8]

supernatant after a 24 h culture at 55 °C in a MSV mineral medium (supplemented with peptone or without). In contrast, for both media, a BTA-hydrolyzing activity was observed (monitored by clear zone test) when BTA films were added to the cultures. Thus, it can be concluded that the addition of peptone stimulates the growth of the microorganisms but does not directly induce the secretion of the BTA hydrolase.

It is obvious that the completely insoluble copolyester BTA stimulates the formation of the enzyme. Lin and Kolattukundy[16] proposed a mechanism for the regulation of a cutinase from *Fusarium solani f. pisi*, where the organism continuously produces a small amount of the enzyme (basal expression). If the insoluble substrate cutin is present, low concentrations of degradation products are released which stimulate the increased formation of the extracellular enzyme. The intensity of the stimulation has been reported to be depending on the structure of the depolymerization product. For the BTA hydrolase from *T. fusca*, no activity could be detected (by clear zone test) in MSV/peptone (0.1%) media supplemented with either 0.02% to 0.2% of terephthalic acid, adipic acid, or 1,4-butanediol (24 h culture at 55 °C), but it was shown by Gouda et al.[13] that these monomers of the BTA copolyester inhibit at higher concentrations the production of the enzymes. Furthermore, it was found that the amount of the enzyme produced apparently depends on the amount of the copolyester BTA used as inducer. The effects were discussed to be the result of a complex mechanism including induction, enzyme inhibition and adsorption as well as desorption of the BTA hydrolase at the hydrophobic BTA surface.

To identify the structure of the molecules which induce expression and secretion of BTA hydrolase, various esters were tested with regard to their inducing effect. The results are shown in Table 1.

Obviously, these findings must be evaluated in view of a complex mechanism of microbial polyester cleavage including several phenomena such as covering the polymer surface by microbes which induces enzyme production followed by its secretion.

The BTA copolyester induces the enzyme independent of the surface area (the surface area of the insoluble substrate can be regarded as an analogue to a substrate concentration). In contrast to the monomers, ester containing oligomers of the copolyester (BT and BTBTB; B = 1,4-butanediol and T = terephthalic acid) induce the enzyme. The same is observed for the diester dibutyl terephthalate, whereas with dimethyl terephthalate no activity was found. Applying phthalate esters instead of derivates of terephthalic acid, also resulted in no detectable enzyme production.

However, the induction of the enzyme is not limited to aromatic structures as present in the BTA copolyesters, since also with the aliphatic polyester poly(ϵ-caprolactone (PCL) an enzyme production was achieved.

Purification. To isolate and purify the extracellular BTA degrading enzyme, combinations of concentration steps (ammonium sulfate precipitation, ultrafiltration) and chromatographic techniques such as ion exchange (IEC) and hydrophobic interaction chromatography (HIC) were studied.

Precipitation of the BTA hydrolase from the culture medium was complete above an ammonium sulfate concentration of 50% of saturation, but only a very limited amount of enzyme could be redissolved from the precipitate by repeated washing with a 20 mM citrate buffer (pH 4.4) or a 20 mM Tris/HCl buffer (pH 9.1). More satisfying was the concentration of the culture supernatant by ultrafiltration (cellulose acetate membrane with a cut off of 10 kDa). However, a loss of enzyme activity was observed with this method as well, which can be attributed to nonspecific, irreversible adsorption of the BTA hydrolase on the membrane. The reduction of the yellowish color of the medium and SDS gel electrophoresis showed that low molecular weight components, probably originating from the peptone in the medium, were separated from the enzyme. The balance of the ultrafiltration process for the enzyme is presented by Gouda et al.[13]

After concentration of the culture medium, chromatographic methods were checked for further purification. For anion-exchange chromatography (IEC), Q-Sepharose as the stationary phase was tested. Enzymes hydrolyzing BTA adsorbed at pH > 9 and for the elution a pH-shift (pH 9−7) showed better results than a salt gradient (0−1 M NaCl). In any case, significant BTA hydrolase activity was found in the break through volume. In the active peak still a number of proteins in the range between 15 and 30 kDa could be observed by SDS gel electrophoresis.

For cation-exchange materials (S−Sepharose, Uno), an adsorption of active proteins could be achieved below pH 4. Here the elution was preferably performed by a salt gradient (0−1 M NaCl). Especially, the continuous-bed material Uno allowed the application of high elution rates. No significant activity was observed in the break through volume and during elution BTA hydrolase activity was found in one single peak. The elution profile for a purification on an Uno column is shown in Figure 2. Gel electrophoresis showed (Figure 4) that low molar mass proteins had separated from the BTA hydrolase, but still several bands between 15 and 30 kDa appeared in the elution peak exhibiting BTA hydrolase activity.

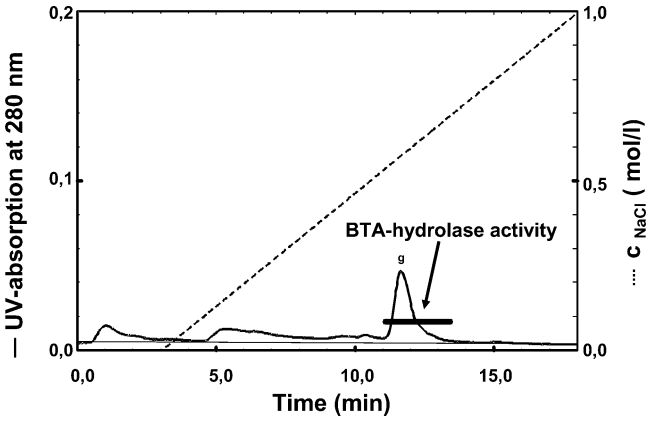

Figure 2. Elution profile for a BTA hydrolase purification with an Uno-S1 cation-exchange column. The black bar indicates the fractions with hydrolase activity. Chromatograpic conditions: start buffer 20 mM citric buffer; elution with a linear gradient of 1 M NaCl solution; sample: 2 mL culture supernatant, redissolved after Na_2SO_4 precipitation, elution rate 2 mL/min; column volume 1.3 mL; T: room temperature.

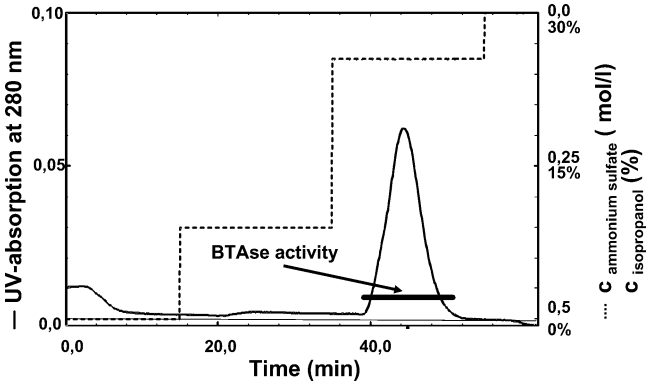

Figure 3. Elution profile for a BTA hydrolase purification with a phenylsepharose CL-4B hydrophobic interaction chromatography column. The black bar indicates the fractions with hydrolase activity. Chromatographic conditions: start buffer 0.5 mM Na_2SO_4 in 20 mM phosphate buffer at pH 7.1; elution with a step gradient of 30% 2-propanol in 20 mM phosphate buffer; sample: 4.7 mL sample of fraction from a cationic exchange chromatography (Uno-S1 column), elution rate 0.3 mL/min; column volume 1.14 mL; T: room temperature.

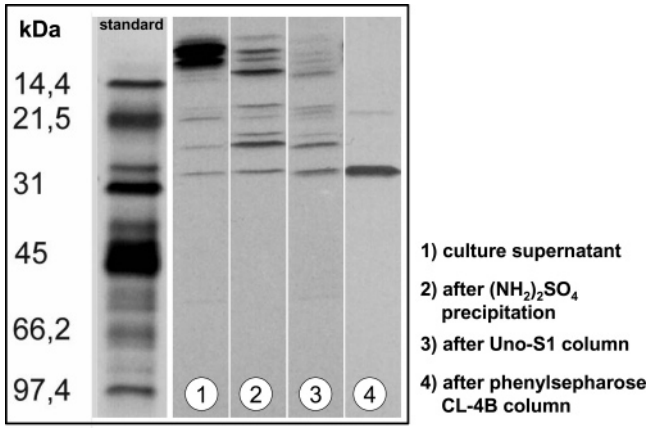

Figure 4. SDS gel chromatograms of purification steps during BTA hydrolase purification (silver staining).

Due to the fact that the BTA degrading enzyme must be able to interact with hydrophobic surfaces, the method of hydrophobic interaction chromatography was suspected to be a reasonable way for further enzyme purification.

The BTA hydrolase binds on phenylsepharose in a 0.5 M ammonium sulfate solution. For elution a gradient from 0.5 M → 0 M ammonium sulfate and 0 → 30% 2-propanol was chosen. No significant differences in the adsorption behavior were observed with phenylsepharose, butylsepharose, and octylsepharose, but no adsorption could be achieved with unmodified sepharose. Thus, a real hydrophobic interaction mechanisms is likely responsible for adsorption. As an example, an elution profile on a phenylsepharose CL-4B column with a step gradient for elution is shown in Figure 3.

BTA hydrolase activity was only observed in one relative sharp peak. One major band between 25 and 30 kDa appeared on SDS gels with some minor impurities (Figure 4).

Hence, the combination of cation exchange and hydrophobic interaction chromatography is proposed as a suitable route of purifying the BTA hydrolase. In the purification process performed with $(NH_4)_2SO_4$ precipitation/Uno-S1 column/phenylsepharose column, an increase of the specific activity by a factor of 110 was achieved with a yield of 14% in activity. The entire balance of the purification procedure is shown in Table 2.

Biochemical Characterization. The actinomycete *Thermobifida fusca* (former name *Thermomonospora fusca*) is a thermophilic organism with a maximum growth rate at 55 °C.[11] Accordingly, the secreted extracellular enzyme exhibits its maximum activity at elevated temperatures. In Figure 5, the dependence of the degradation of a BTA40:60 copolyester on the temperature is shown for the purified hydrolase.

At 65 to 70 °C the copolyester is most rapidly degraded by the enzyme. This maximum is about 10−15 °C higher than the temperature of maximum growth rate of the organisms. This behavior is not unusual for extracellular enzymes[17] and described in the literature, e.g., also for xylanases,[18−20] lipases,[21−24] and PHB-depolymerases.[25] However, the influence of the temperature on the mobility of the macromolecular substrate BTA, which has been described by Marten et al.,[9] can superimpose the effect of enzyme activity (regarded as ester cleavage activity on low molecular weight substances such as oils or fats) and, hence, results in an apparent higher temperature maximum than it would be observed for a low molecular weight substrate. The rapid decrease in activity at temperatures higher than 70 °C is probably caused by the denaturation of the enzyme, since, e.g., at 65 °C, denaturation is significant within the period of time needed to measure the activity (30 min).

The purified enzyme losses about 85% of its activity within 30 min at a temperature of 70 °C. The temperature stability of a crude and an ultrafiltrated enzyme solution was characterized by Gouda et al.[13] in detail. Here it was found that about 60% of the activity remained after one week storage in a crude enzyme solution at room temperature but drops to nearly zero within 1 h at 70 °C. Ultrafiltrated solutions with higher enzyme concentrations were reported to be slightly more stable than the crude solution.

The dependence of the enzyme activity on the pH is shown in Figure 6. The maximum activity was observed between pH 6.0 and 6.5, whereby the peaks obviously shift toward neutral pH if the ionic strength of the solution decreases.

Table 2. Balance of the BTA hydrolase Purification

purification step	total volume (mL)	total protein (mg)	total activity (U)[a]	specific activity (U/mL)	activity yield (%)
culture broth	1600	5520	18133	3.3	100
after Na_2SO_4 precipitation	107	70	6777	97	37
after ion exchange chrom. (UNO−S1 column)	42	21	4584	218	25
after hydrophobic interaction chrom. (CL-4HB column)	9.5	7	2521	360	14

[a] Based on tributyrin as substrate; one unit is defined a activity cleaving 1 μmol esters per min.

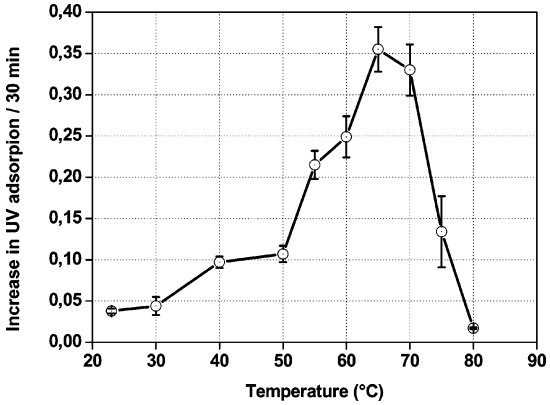

Figure 5. Temperature dependence of the BTA hydrolase activity toward BTA40:60 films. Activity was determined with the UV test described in "Materials and Methods". The activity was calculated from OD differences at the first 30 min of incubation.

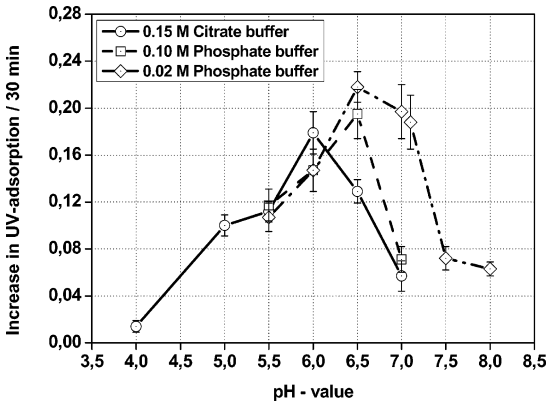

Figure 6. pH dependence of the BTA hydrolase activity toward BTA40:60 films. Activity was determined with the UV test described in "Materials and Methods". The activity was calculated from OD differences at the first 30 min of incubation at a temperature of 55 °C.

The BTA degrading enzyme is able to cleave ester bonds in low molecular weight compounds, as well. Figure 7 shows the results on ester cleavage of triglycerides with different chain length of the fatty acid.

For the triglycerides with short chain length of the fatty acids (triacetin C = 2, tributyrin C = 4, and tricaproin C = 6), the BTA hydrolase exhibits an almost equal activity, whereas for longer fatty acids (tricaprylin C = 8, tricaprin C = 10, trilaurin C = 12, and triolein C = 18) the ester cleavage rate decreases significantly with the number of carbon atoms in the fatty acids.

Also for polyesters, the enzyme exhibits a quite broad substrate spectrum. Besides the aliphatic−aromatic copolyesters also a number of aliphatic polyesters including poly(ϵ-caprolactone) (PCL), poly(trimethylene brassylate) (SP3/13), the commercial aliphatic, biodegradable plastics

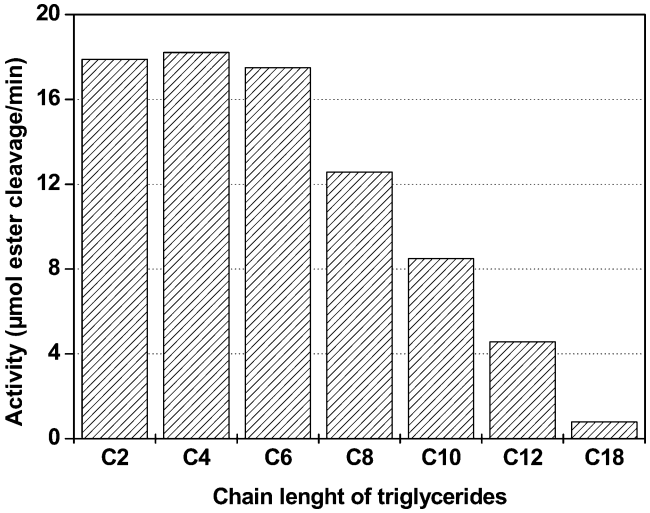

Figure 7. Influence of the chain fatty acid chain length on the hydrolysis of different triglycerides with BTA hydrolase at 55 °C. 20 μg enzyme was added to 6 mL of substrate solution.

Table 3. Characteristic Data for the BTA hydrolase from *Thermobifida fusca* DSM 43793

molar mass$_{\text{gel electroporesis}}$	27.4 kDa
molar mass$_{\text{amino acid sequence}}$	28.2 kDa
p*I* value	pH 6.43
temperature optimum $_{\text{BTA degradation}}$	65−70 °C
pH optimum $_{\text{BTA degradation}}$	
0.15 M citrate buffer	pH 6.0
0.10 M citrate buffer	pH 6.5
0.02 M phosphate buffer	pH 6.5−7.0
substrates	triglycerides
	aliphatic polyesters
	aliphatic−aromatic copolyesters

Bionolle, and the aliphatic polyester amide Bayer TIR 1874 proved to be cleavable by the BTA hydrolase. In contrast, the natural polyester poly(β-hydroxy butyrate) (PHB) was not attacked by this enzyme.

In Table 2, the main characteristic data for the enzyme are compiled.

Amino Acid Sequence. The amino acid sequence of the purified enzyme was determined from the corresponding band of the SDS gel electrophoresis. The sequence is shown in Table 3.

The enzyme consists of 261 amino acids and was classified as a serine hydrolase. It exhibits a 65% similarity to a triacylglycerol lipase from *Streptomyces albus* G[26] and a 62% similarity to a triacylglycerol acylhydrolase from *Streptomyces sp*. M11[27] (SP-TREMBL data bank release 7.0, 08.1998). The active site of serine hydrolases typically contains a highly conserved **-G**-X_1-**S**-X_2-**G**- sequence which has been found to be the **-G**-H-**S**-M-**G**- sequence in the BTA hydrolase (serine at position 132 of the enzyme).

Table 4. Amino Acid Sequence of the BTA Hydrolase and Alinement with the Sequences of a Triacylglycerol Lipase from *Streptomyces albus* G and a Triacylglycerol *Streptomyces sp.* M 1[a]

Triacylglycerol lipase [1)]	D	N	P	Y	E	R	G	P	A	P	T	R	A	S	I	E	A	P	R	G	P	Y	A	V	S	Q	T	S	V	S
Triacylglycerol acylhydrolase [2)]	A	N	P	Y	E	R	G	P	A	P	T	N	A	S	I	E	A	S	R	G	P	Y	A	T	S	Q	T	S	V	S
BTA - hydrolase	**A**	**N**	**P**	**Y**	**E**	**R**	**G**	**P**	**N**	**P**	**T**	**D**	**A**	**L**	**L**	**E**	**A**	**S**	**S**	**G**	**P**	**F**	**S**	**V**	**S**	**E**	**E**	**N**	**V**	**S**
									10										20										30	
Triacylglycerol lipase [1)]	S	L	V	V	S	G	F	G	G	G	T	I	Y	Y	P	T	S	T	G	D	G	T	F	G	A	V	V	V	T	P
Triacylglycerol acylhydrolase [2)]	S	L	V	A	S	G	F	G	G	G	T	I	Y	Y	P	T	S	T	A	D	G	T	F	G	A	V	V	I	S	P
BTA - hydrolase	**R**	**L**	**S**	**A**	**S**	**G**	**F**	**G**	**G**	**G**	**T**	**I**	**Y**	**Y**	**P**	**R**	**E**	**-**	**-**	**N**	**N**	**T**	**Y**	**G**	**A**	**V**	**A**	**I**	**S**	**P**
									40										50										60	
Triacylglycerol lipase [1)]	G	F	T	A	T	E	S	S	M	A	W	L	G	P	R	L	A	S	Q	G	F	V	V	F	T	I	D	T	L	T
Triacylglycerol acylhydrolase [2)]	G	F	T	A	Y	Q	S	S	I	A	W	L	G	P	R	L	A	S	Q	G	F	V	V	F	T	I	D	T	N	T
BTA - hydrolase	**G**	**Y**	**T**	**G**	**T**	**E**	**A**	**S**	**I**	**A**	**W**	**L**	**G**	**E**	**R**	**I**	**A**	**S**	**H**	**G**	**F**	**V**	**V**	**I**	**T**	**I**	**D**	**T**	**I**	**T**
									70										80										90	
Triacylglycerol lipase [1)]	T	L	D	Q	P	D	S	R	G	R	Q	M	L	A	A	L	D	Y	L	T	E	R	-	-	S	S	A	R	T	R
Triacylglycerol acylhydrolase [2)]	T	L	D	Q	P	D	S	R	G	R	Q	L	L	S	A	L	D	Y	L	T	Q	R	-	-	S	S	V	R	T	R
BTA - hydrolase	**T**	**L**	**D**	**Q**	**P**	**D**	**S**	**R**	**A**	**E**	**Q**	**L**	**N**	**A**	**A**	**L**	**N**	**H**	**M**	**I**	**N**	**R**	**A**	**S**	**S**	**T**	**V**	**R**	**S**	**R**
									100										110										120	
Triacylglycerol lipase [1)]	I	D	G	T	R	L	G	V	I	G	H	S	M	G	G	G	G	T	L	E	A	A	K	S	R	P	S	L	K	A
Triacylglycerol acylhydrolase [2)]	V	D	A	T	R	L	G	V	M	G	H	S	M	G	G	G	G	S	L	E	A	A	K	S	R	T	S	L	K	A
BTA - hydrolase	**I**	**D**	**S**	**S**	**R**	**L**	**A**	**V**	**M**	**G**	**H**	**S**	**M**	**G**	**G**	**G**	**G**	**T**	**L**	**R**	**K**	**A**	**S**	**Q**	**R**	**P**	**D**	**L**	**K**	**A**
									130										140										150	
Triacylglycerol lipase [1)]	A	I	P	L	T	P	W	N	L	D	K	T	W	P	E	V	T	T	P	T	L	V	V	G	A	D	G	D	T	V
Triacylglycerol acylhydrolase [2)]	A	I	P	L	T	G	W	N	T	D	K	T	W	P	E	L	R	T	P	T	L	V	V	G	A	D	G	D	T	V
BTA - hydrolase	**A**	**I**	**P**	**L**	**T**	**P**	**W**	**H**	**L**	**N**	**K**	**N**	**W**	**S**	**S**	**V**	**T**	**V**	**P**	**T**	**L**	**I**	**I**	**G**	**A**	**D**	**L**	**D**	**T**	**I**
									160										170										180	
Triacylglycerol lipase [1)]	A	P	V	A	T	H	A	K	P	F	Y	S	S	L	P	S	S	T	D	R	A	Y	L	E	L	N	N	A	T	H
Triacylglycerol acylhydrolase [2)]	A	P	V	A	T	H	S	K	P	F	Y	E	S	L	P	G	S	L	D	K	A	Y	L	E	L	R	G	A	S	H
BTA - hydrolase	**A**	**P**	**V**	**A**	**T**	**H**	**A**	**K**	**P**	**F**	**Y**	**N**	**S**	**L**	**P**	**S**	**S**	**I**	**S**	**K**	**A**	**Y**	**L**	**E**	**L**	**D**	**G**	**A**	**T**	**H**
									190										200										210	
Triacylglycerol lipase [1)]	F	A	P	N	L	S	N	T	T	I	A	K	Y	S	V	S	W	L	K	R	F	I	D	D	D	T	R	Y	E	Q
Triacylglycerol acylhydrolase [2)]	F	T	P	N	T	S	D	T	T	I	A	K	Y	S	I	S	W	L	K	R	F	I	D	S	D	T	R	Y	E	Q
BTA - hydrolase	**F**	**A**	**P**	**N**	**I**	**P**	**N**	**K**	**I**	**I**	**G**	**K**	**Y**	**S**	**V**	**A**	**W**	**L**	**K**	**R**	**F**	**V**	**D**	**N**	**D**	**T**	**R**	**Y**	**T**	**Q**
									220										230										240	
Triacylglycerol lipase [1)]	F	L	C	P	L	P	V	P	D	R	-	-	D	I	E	E	Y	R	G	T	C	P	L	G	G					
Triacylglycerol acylhydrolase [2)]	F	L	C	P	I	P	R	P	S	L	-	-	T	I	A	E	Y	R	G	T	C	P	H	T	S					
BTA - hydrolase	**F**	**L**	**C**	**P**	**G**	**P**	**R**	**D**	**G**	**L**	**F**	**G**	**E**	**V**	**E**	**E**	**Y**	**R**	**S**	**T**	**C**	**P**	**F**							
									250										260											

[a] The gray boxes indicate differences between the sequences of the three hydrolases compared in the alinement. [1)] Triacylglycerol lipase from *Streptomyes albus* G.[26] [2)] Triacylglycerol acylhydrolase from *Streptomyces sp.* M11.[27]

The fraction of hydrophobic amino acids (Ala, Val, Leu, Ile, Pro, Met, Phe, Trp) is 42% high compared with other serine hydrolases.[24,28] The hydrophobicity of the BTA hydrolase explains its tendency to adsorb on ultrafiltration membranes and the problems in resolving the $(NH_4)_2SO_4$ precipitate.

Classification of the Enzyme. Based on the amino acid sequence, the BTA hydrolyzing enzyme was identified to be a serine hydrolase. Subgroups of the serine hydrolases are esterases, lipases, and cutinases. Since the BTA hydrolase from *Thermobifida fusca* (TfH) is able to attack triglycerides such as tributyrin (Figure 7) the enzyme obviously does not belong to the group of esterases which are only able to cleave ester substances dissolved in water. The amino acid sequence showed most similarities to two lipases (see above) and from this fact it could be supposed that the BTA hydrolase is a lipase. However, detailed experiments on the hydrolysis of polyesters with the BTA hydrolase in comparison with different lipases revealed significant differences in the behavior of these enzymes (Figures 8 and 9).

In Figure 8, the degradation of films of the polyester SP4/6 with lipases from *Pseudomonas sp.* (PsL) and *Aspergillus oryzae* (AoL) is compared with the hydrolysis of the films with the BTA hydrolase. Both lipases were able to attack the ester bonds at the surface of the solid and insoluble polyester, but the ester cleavage stopped for both enzymes when 40% of the ester bonds present in the films had been degraded. This effect cannot be attributed to an inactivation of the enzymes, since an addition of fresh PsL after 4 h did not result in a further cleavage of esters. At the time the plateau is reached, the polyester films were already totally disintegrated and transformed into water soluble degradation

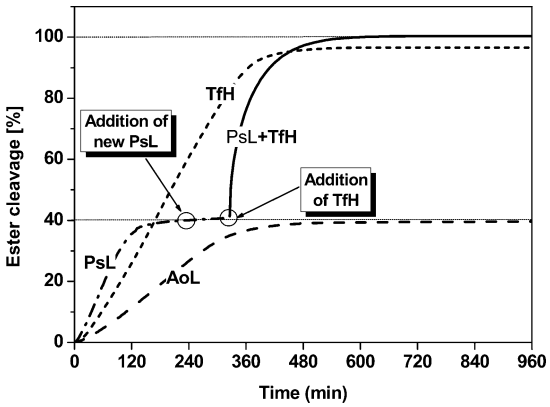

Figure 8. Comparison of the degradation of SP4/6 films hydrolase from *T. fusca* (TfH) and lipases from *Aspergillus oryzae* (Aol) and *Pseudomonas sp.* (PsL) at 40 °C. 2 films, ø: 0.9 cm, thickness 110 μm; titration of released acids in 6 mL of 0.9 M NaCl; enzyme addition PsL, 1.024 mg (with approximately 40% protein content); AoL, 1.665 mg (with approximately 60% protein content); TfH, 10 mg (with approximately 5% protein content).

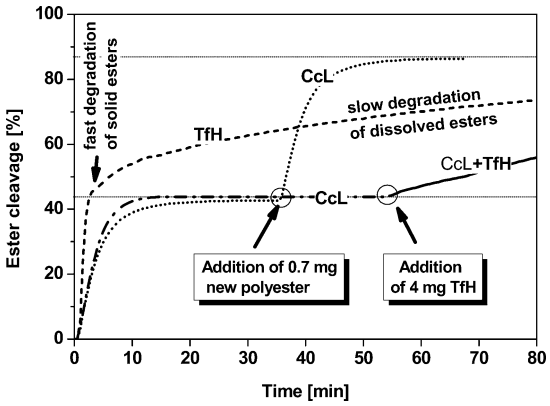

Figure 9. Comparison of the degradation of SP4/6 nanoparticles (0.7 mg; ø: 210 nm) with the hydrolase from *T. fusca* (TfH) and a lipase from *Candida cylindracea* (CcL) at 40 °C.

intermediates. These intermediates still contain ester bonds, which cannot be attacked by the lipases, since lipases need a hydrophobic surface to be activated by a conformational change. The lipases act on the surface of the polyesters and randomly reduce the chain length of the polyester chains until they are short enough to become water soluble. The height of the plateau usually does not depend of the kind of lipase but is influenced predominantly by the structure of the polymer, determining the solubility of the intermediates.[29] In contrast to the lipases, the hydrolase from *T. fusca* (TfH) is able to cleave almost 100% of the available esters in the polymer and, hence, must also be able to cleave dissolved esters. Therefore, TfH does not necessarily need a hydrophobic surface to be active, as can be demonstrated by adding TfH to a solution containing the degradation intermediates of a polyester depolymerization with PsL (Figure 8). Although the polyester film has entirely been dissolved and no hydrophobic solid surface is present, TfH cleaves the ester bond in the dissolved intermediates to 100%.

A corresponding behavior is observed when degrading polyester nanoparticles instead of films (Figure 9) with a lipase from *Candida cylindracea* (CcL) (the activity of PsL and AoL are too high for experiments with nano particles). Again only a 40% ester cleavage is obtained with CcL. Addition of fresh polymer in the plateau phase demonstrates again that the stagnation in ester cleavage is not caused by an inactivation of the lipase since again approximately 40% of the added ester bonds are cleaved. In contrast to the degradation experiment with the SP4/6 films, two phases in the degradation kinetics with TfH can be observed. Due to the larger specific surface area (and a changed crystallinity), the ester cleavage in the presence of the solid polyester is very fast, followed then by a much slower hydrolysis of the esters in the dissolved intermediates. From this observation, it seems a likely supposition that the activity of TfH is enhanced when coming into contact with the hydrophobic surface of the polyester (as specific for lipases) but exhibits also a certain activity toward dissolved esters (as specific for esterases). The cleavage rate in the second phase is comparable to that obtained by adding TfH to a solution containing the intermediates of a degradation with CcL. For the film degradation (Figure 8), the higher activity toward solid, hydrophobic surfaces was compensated by the relatively small surface area available for the polyester degradation.

Since the TfH obviously exhibits neither only the typical behavior of a lipase, which requires surface activation, nor only that of an esterase, it could be anticipated to classify the TfH as a cutinase. In the literature, enzymes are classified as cutinases if they are able to cleave esters of the natural polyester cutin and if they do not require a surface activation on hydrophobic surfaces.[30] Nishida et al.[31] found that various plant pathogenic microorganisms are able to degrade the aliphatic polyester poly(ϵ-caprolactone) and suspected that the extracellular enzymes used by these organisms could be cutinases, since these enzymes are discussed to be closely related to the pathogenity mechanism.[30] In a paper published by Murphy et al.,[7] it was then shown that PCL was degraded by an extracellular enzyme of a *Fusarium* strain which degraded cutin as well. A central argument to declare a cutinase as PCL degrading enzyme was the simultaneously occurring esterase activity besides the PCL degrading activity as well as the induction of the enzyme by cutin hydrolysates. The authors also draw some parallels between cutin degradation products and a trimer of PCL. Further evidence for TfH being a cutinase may be drawn from a recent publication of Fett et al.[32] where the production of a cutinase by a *Thermobifida fusca* strain (ATCC 27730) is described.

However, despite the fact that TfH seems to behave between a lipase and a esterase, the high activity observed toward the solid polyester surface points to a surface activation mechanism, whereas the missing of such a surface activation is assumed as a characteristics of cutinases. As shown in Figure 10, TfH is able to attack cutin, which was purified from apple skin.

However, also the lipase from *Pseudomonas sp.* (PsL) attacks cutin at a comparable rate, while an esterase (BsE) did not hydrolyze the insoluble substrate at all. These results indicate that the property of cutin degradation alone is not a suitable criteria to discriminate among different hydrolases, specifically lipases and cutinases. Obviously, as demonstrated by the studies with various substrates and hydrolytic enzymes the hydrolase isolated from *T. fusca* exhibits hydrolytical

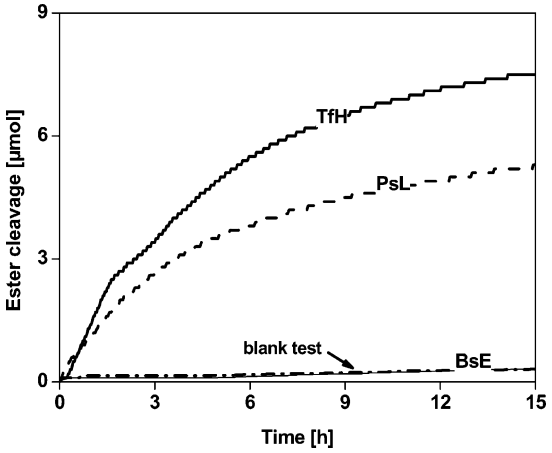

Figure 10. Degradation of cutin by the BTA hydrolase (TfH), a lipase *from Pseudomonas sp.* (PsL) and an esterase from *Bacillus stearothermophilus* (BsE) at 40 °C. Determination of released acids by titration in 6 mL of 0.9% NaCl with 10 mg cutin (grinded powder). Addition of enzyme TfH, 5 mg; PsL, 1 mg; BsE, 1.5 mg.

properties typical for lipases, cutinases, and esterases as well. Hence, the conventional strict discrimination among substrate specific enzymatic functions appears obsolete and can hardly be maintained further on.

Conclusions

The thermophilic hydrolase isolated from *Thermobifida fusca* was obtained in the course of a screening for polyester degrading organisms. Thus, it was not surprising to find, for the extracellular enzyme, characteristics of a lipase since polyesters are strongly hydrophobic and water insoluble substrates. However, the presence of a certain esterase activity, able to cleave also dissolved esters, is not usual for lipases but sometimes attributed to enzymes classified as cutinases. In our opinion, the characteristics of enzymes described as cutinases and their discrimination from lipases are not very clear, and we abstain at this stage from identify the BTA hydrolase as a cutinase. In any case, the behavior of the BTA lipase lies between a typical lipase and an esterase.

From the structural viewpoint it could be supposed, that the active site of the BTA hydrolase is not submerged deeply into the three-dimensional protein structure, which would facilitate the attack of ester bonds embedded in a rigid polymer chain in, e.g., crystals of aliphatic−aromatic copolyesters. Possibly, the hydrolase does not possess an extended lid covering the active center in the not activated state. However, this questions can only finally answered if the three-dimensional structure of the protein has been determined.

References and Notes

(1) Mukai, K.; Doi, Y. *Prog. Ind. Microbiol.* **1995**, *32*, 189.
(2) Braunegg, G.; Lefebvre, G.; Genser, K. F. *J. Biotechnol.* **1998**, *65*, 127.
(3) Müller, R.-J.; Kleeberg, I.; Deckwer, W.-D. *J. Biotechnol.* **2001**, *86* (2), 87.
(4) Jäger, K.-E.; Steinbüchel, A.; Jendrossek, D. *Appl. Environ. Microbiol.* **1995**, *61* (8), 3113.
(5) Jendrossek, D.; Handrick, R. *Annu. Rec. Microbiol.* **2002**, *56*, 403.
(6) Tokiwa, Y.; Ando, T.; Suzuki, T.; Takeda, K. *ACS Symp. Ser. (Agric. Synth. Polym.: Biodegrad. Util.)* **1990**, *433*, 136.
(7) Murphy, C. A.; Cameron, J. A.; Huang, S. J.; Vinopal, R. T. *Appl. Environ. Microbiol.* **1996**, *62* (2), 456.
(8) Marten, E. Dissertation TU−Braunschweig, Germany, 2000; http://opus.tu-bs.de/opus/volltexte/2000/136.
(9) Marten, E.; Müller, R.-J.; Deckwer, W.-D. *Polym. Degrad. Stab. 80* (3), **2003**, 485.
(10) Welzel, K. Dissertation TU−Braunschweig, Germany, 2003; http://opus.tu-bs.de/opus/volltexte/2003/460.
(11) Kleeberg, I.; Hetz, C.; Kroppenstedt, R. M.; Müller, R.-J.; Deckwer, W.-D. *Appl. Environ. Polym. Degrad.* **1998**, *64* (5), 1731.
(12) Kleeberg, I. Dissertation TU−Braunschweig, Germany, 1999; http://opus.tu-bs.de/opus/volltexte/2000/90.
(13) Gouda, M. K.; Kleeberg, I.; Van den Heuvel, J.; Müller, R.-J.; Deckwer, W.-D. *Biotechnol. Prog.* **2002**, *18* (5), 927.
(14) Walton, T. J.; Kolattukudy, P. E. *Biochemistry* **1972**, *11* (10), 1885.
(15) Schlegel, H. G. *Allgemeine Mikrobiologie*; Gerorg Thieme Verlag: Stuttgart, Germany, 1992.
(16) Lin, T. S.; Kolattukudy, P. E. *Plant Pathol.* **1980**, *17* (1), 1.
(17) McCarthy, A. J.; Ball, A. S.; Bachmann, S. L. Biology of actinomycetes. In *Proceedings of seventh international symposium on biology of actinomycetes*; Okami, Y., Beppu, T., Ogawara, H., Eds.; Japan Scientific Societies Press: Tokyo, 1988; p 263.
(18) Holtz, C.; Kaspari, H.; Klemme, J. H. Production and properties of xylanases from thermophilic actinomycetes. *Int J. General Mol. Microbiol.* **1991**, *59*, 1−7.
(19) Kluepfel, D.; Sharek, F.; Mondou, F.; Morosoli, R. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 230.
(20) McCarthy, A. J.; Peace, E.; Broda, P. *Appl. Microbiol. Biotechnol.* **1985**, *21*, 238.
(21) Liu, W. H.; Beppu, T.; Arima, K. *Agr. Biol. Chem.* **1972**, *36* (11), 1919.
(22) Muroka, T. Ando, T.; Okuda, H. *J. Biochem.* **1982**, *92*, 1933.
(23) Sugihara, A.; Tani, T.; Tominaga, Y. *J. Biochem.* **1991**, *109*, 211.
(24) Schmidt-Dannert, C.; Sztajer, H.; Stöcklein, W.; Menge, U.; Schmidt, R. D. *Biochim. Biophys. Acta* **1994**, *1214*, 43.
(25) Müller, B.; Jendrossek, D. *Appl. Microbiol. Biotechnol.* **1993**, *38*, 487.
(26) Cruz, H.; Pérez, C.; Wellington, E.; Castro, C.; Severin-González, L. *Gene* **1994**, *144*, 141.
(27) Pérez, C.; Juárez, K.; Garcia-Castels, E.; Soberón, G.; Severin-González, L. *Gene* **1993**, *123*, 109.
(28) Ziemann-Nöthe, A. Dissertation TU−Braunschweig, Germany, 1995.
(29) Walter, T.; Augusta, J.; Müller, R.-J.; Widdecke, H.; Klein, *J. Enzyme Microbiol. Technol.* **1995**, *17*, 218.
(30) Carvalho, C. M.; Aires-Barros, M. R.; Cabral, J. M. S. *Biotechnol. Bioeng.* **1999**, *66* (1), 17.
(31) Nishida, H.; Tokiwa, Y. *Chem. Lett.* **1994**, *8*, 1547.
(32) Fett, W. F.; Wijey, C.; Moreau, R. A.; Osman, S. F. *J. Appl. Microbiol.* **1999**, *86* (4), 561.

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