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A Novel β-Peptidyl Aminopeptidase (BapA) from Strain 3-2W4 Cleaves Peptide Bonds of Synthetic β-Tri- and β-Dipeptides

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A novel bacterial strain that was capable of growing on the β-tripeptide H-βhVal-βhAla-βhLeu-OH as the sole carbon and nitrogen source was isolated from an enrichment culture. On the basis of physiological characterization, partial 16S rRNA sequencing, and fatty acid analysis, strain 3-2W4 was identified as a member of the family *Sphingomonadaceae*. Growth on the β-tripeptide and the β-dipeptide H-βhAla-βhLeu-OH was observed, and emerging metabolites were characterized. Small amounts of a persisting metabolite, the N-acetylated β-dipeptide, were identified in both media. According to dissolved organic carbon measurements, 74 to 80% of the available carbon was dissimilated. The β-peptide-degrading enzyme was purified from the crude cell extract of cells from strain 3-2W4 grown on complex medium. The enzyme was composed of two subunits, and the N-terminal sequences of both were determined. With this information, it was possible to identify the complete nucleotide sequence and to deduce the primary structure of the gene *bapA*. The gene encoded a β-peptidyl aminopeptidase (BapA) of 402 amino acids that was synthesized as preprotein with a signal sequence of 29 amino acids. The enzyme was cleaved into two subunits (residues 30 to 278 and 279 to 402). It belonged to the N-terminal nucleophile (Ntn) hydrolase superfamily.

β-Amino acids and their derivatives occur as building blocks of many natural products (30). For example, they are constituents of naturally occurring peptides, such as bestatin, nodularin, and microcystin, and of nonpeptidic, pharmaceutically active substances, such as paclitaxel (Taxol). However, naturally occurring peptides that consist exclusively of β-amino acids are not known so far. In 1996, β-peptides consisting of β-amino acid residues with proteinogenic side chains were synthesized and described for the first time (32). Since then, much knowledge about the properties and possible applications of this class of substance has accumulated. High stability against peptidases and proteases (10, 14–16, 20, 29, 32) is one of the prominent characteristics of β-peptides. Already, very early investigations by Aberhalden et al. suggested that peptide bonds between α- and β-amino acids are resistant to enzymatic hydrolysis (1, 2). During recent years, this statement has been validated by a series of in vitro studies. Very detailed tests have shown that a variety of peptides composed of β-amino acids were stable against many different commercially available proteases and peptidases (10, 29). Furthermore, first studies on the stability of β-peptides in vivo have shown that virtually no degradation was observed when such peptides were administered to rats (42, 43). Some β-peptides possess antimicrobial activities (4, 5, 25, 38), bind to the human somatostatin receptor (12, 21), function as inhibitors of human immunodeficiency virus type 1 replication (39), and inhibit p53-hDM2 interaction (18). Cationic β-peptides cross bacterial and mammalian cell membranes and can be considered a new group of cell-penetrating peptides (13, 26, 31). Because they are able to form different helices (11) and to mimic amphipathic α-peptidic helices and turns (7, 41), β-peptides have a high potential for being developed as novel pharmaceutically active substances that function as stable peptidomimetics (21, 30, 34).

However, the reported extraordinary metabolic stability of β-peptides leads to concerns with regard to a general metabolic inertness of this class of compound. Previously, we have shown the general biodegradability of the nonnatural β-peptides H-βhVal-βhAla-βhLeu-OH (nomenclature according to Seebach et al. [30]) and H-βhAla-βhLeu-OH (28). Considering ecological aspects, this fact is of great importance, because β-peptides applied in medicinal chemistry or as materials eventually will enter the environment.

Here, we describe the isolation and characterization of strain 3-2W4, which is able to utilize the β-peptides H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH as sole carbon and energy sources. A β-peptidyl aminopeptidase able to cleave these β-peptides was identified and purified, and the corresponding gene was identified and sequenced.

MATERIALS AND METHODS

Chemicals. The preparation and characterization of the β-tripeptide H-βhVal-βhAla-βhLeu-OH and of the β-dipeptide H-βhAla-βhLeu-OH have been described previously (28). For the preparation of larger amounts of β-tripeptide required for the present investigation, the procedure was somewhat modified. The solution synthesis started with H-βhLeu-O-Bn, which was coupled with Boc-βhAla-OH to give the protected β-dipeptide. Boc deprotection and coupling with Cbz-βhVal-OH (35) provided Cbz-βhVal-βhAla-βhLeu-OBn. The coupling reagents were *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride and 1-hydroxy-1H-benzotriazole in the presence of *N*-methylmorpholine. Final deprotection by catalytic hydrogenation in methanol furnished the β-tripeptide, which was purified by multiple precipitation, trituration, and washing with mixtures of diethyl ether and methanol. The largest batch provided ca. 12 g of β-tripeptide with a high-pressure liquid chromatography (HPLC) purity of >98%. The following commercial β amino acid derivatives were employed as

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starting materials: Boc-βhAla-OH, Boc-βhLeu-OBn, and Boc-βhVal-OH (for Boc/Cbz protective-group interchange) (Fluka AG, Buchs, Switzerland). The β-peptides were named according to Seebach et al. (30). The N-acetylated β-dipeptide Ac-βhAla-βhLeu-OH was prepared as follows: H-βhAla-βhLeu-OH (18.7 μmol) was dissolved in 0.5 ml of aqueous sodium bicarbonate solution (1 M) and cooled to 0°C. Acetic anhydride solution in acetonitrile (1%, vol/vol; 0.5 ml) was added, and the resulting mixture was allowed to warm up and was then stirred at 23°C for 12 h (33). The reaction mixture was washed once with ethyl acetate, carefully acidified with 1 M HCl, and immediately extracted with ethyl acetate (three times with 2 ml). The combined organic phase was dried over Na_2SO_4 to give analytically pure Ac-βhAla-βhLeu-OH (quantitative yield). The identity of the synthesized product was confirmed by high-resolution mass spectrometry. (See Fig. 4 for the formulae of the three β-peptides.) All other chemicals and reagents used were of analytical grade.

Media and growth conditions. TGY medium and Luria-Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per liter, pH 7.5) served as nonselective complex media. TGY medium, tryptone, and yeast extract were obtained from Biolife (Milano, Italy). For preparation of solid medium, 1.5% (wt/vol) agar was added.

Cells were cultivated aerobically at 25°C on a rotary shaker (170 to 240 rpm). β-Tripeptide minimal medium (TriMM1) and β-dipeptide minimal medium (DiMM1) for enrichment cultures and for isolation of the β-peptide-degrading strain(s) contained 5 mM H-βhVal-βhAla-βhLeu-OH or H-βhAla-βhLeu-OH as the sole source of carbon. Each medium was supplemented with 0.2 mM $CaCl_2$, 2 mM $MgSO_4$, 1 ml/liter of a trace element solution, and 2 ml/liter of a vitamin solution, according to methods outlined in reference 28. Each medium also contained 1 g of NaCl, 12 g of $Na_2HPO_4 \cdot 2H_2O$, 6 g of KH_2PO_4, 0.7 g of $(NH_4)_2SO_4$, and 0.1 g of yeast extract per liter of medium. The β-tripeptide, $CaCl_2$, $MgSO_4$, trace element solution, and vitamin solution were sterilized by filtration and added to the other components, which were sterilized by autoclaving. For experiments with strain 3-2W4, TriMM1 and DiMM1 were changed by omitting $(NH_4)_2SO_4$ and decreasing the concentrations of phosphate salts to 2 g/liter of $Na_2HPO_4 \cdot 2H_2O$ and to 1 g/liter of KH_2PO_4 (TriMM2 and DiMM2).

The susceptibilities of strain 3-2W4 to seven antibiotics were determined in LB broth. Each flask was supplemented with one antibiotic in the following concentrations: streptomycin sulfate (10 and 50 μg/ml), ampicillin (20 and 40 μg/ml), tetracycline (10 and 50 μg/ml), kanamycin (10 and 50 μg/ml), chloramphenicol (25 and 170 μg/ml), rifampin (50 and 150 μg/ml), and gentamicin (50 μg/ml). The flasks were inoculated with 2% (vol/vol) preculture grown in LB broth and monitored for growth after 3 and 5 days.

Strain 3-2W4 was cultivated aerobically in a 2-liter Mini bioreactor (MBR Bioreactor AG, Switzerland) at 25°C. The fermentation was started by adding 1% (vol/vol) inoculum to 1.6 liter of LB medium. The pH was set to 7.5 without further adjustment, and the lower partial O_2 pressure limit did not fall below a value of 40%.

Analytical procedures. (i) HPLC and LC–MS-MS analysis of substrates and metabolites. Supernatants of bacterial cultures were analyzed by reversed-phase HPLC on a Gynkotek HPLC system equipped with an M480 pump, a Gina50 autosampler, and a UVD340 photodiode array detector. Samples were centrifuged for 10 min at 16,000 × *g* and 4°C, and the supernatant was directly injected onto a Nucleosil 100-5 C_4 column (250 by 4 mm; Macherey-Nagel, Düren, Germany). The β-peptides and their derivatives were detected by measuring the UV absorption at 205 nm. Trifluoroacetic acid (0.1%, vol/vol) in water (solvent A) and acetonitrile (solvent B) were used as eluents at a flow rate of 1 ml/min. The program started with a gradient from 10 to 30% solvent B within 8 min, followed by 30% solvent B for 4.8 min, and a second gradient from 30 to 10% solvent B within 1 min. The column was reequilibrated with 10% solvent B for 5 min. Mass spectrometry measurements were performed on an API 4000 liquid chromatography-tandem mass spectrometry (LC–MS-MS) system equipped with an Agilent 1100 LC system (Applied Biosystems, Rotkreuz, Switzerland).

(ii) Ion-exchange chromatography. Nitrate, nitrite, and ammonium ions were measured by ion-exchange chromatography by means of an ED50 electrochemical detector, a GP40 gradient pump, and an IonPac AS11 column (diameter, 4 mm) with an ASRS ultra-II suppressor or an IonPac CS12 column (diameter, 4 mm) with a CSRS ultra-II 4-mm suppressor (Dionex, Olten, Switzerland). For anion exchange, the following flow program was applied at a flow rate of 1 ml/min. The program started with 0.5 mM NaOH for 1.5 min, followed by a gradient from 0.5 to 26 mM NaOH within 9.5 min and a second gradient from 26 mM to 0.5 mM NaOH within 0.5 min. At the end of the program, the column was reequilibrated with 0.5 mM NaOH for 3.5 min. For cation-exchange chromatography, the analytes were isocratically eluted with an 18-mM solution of methanesulfonic acid at a flow rate of 1 ml/min.

(iii) DOC. For the measurement of dissolved organic carbon (DOC), samples were filtered through polyvinylidene difluoride (PVDF) filters (pore size, 0.22 μm) and acidified with HCl (pH 2). Dissolved CO_2 was removed by purging with nitrogen for 6 min before analyzing the sample with a Tocor 2 carbon analyzer (Maihak, Hamburg, Germany).

(iv) Determination of cell dry weight. Cell dry weight was determined by filtering a 10-ml sample through a 0.22-μm PVDF filter, followed by washing with 10 ml of deionized water. The filters were dried at 105°C and cooled in a desiccator, and the difference in weight was determined.

Isolation of metabolites. For identification of the second metabolite, strain 3-2W4 was cultivated in TriMM1 supplemented with 2 g/liter of yeast extract for 11 days. The cells were removed by centrifugation, and the remaining supernatant was filtered through a 0.22-μm PVDF filter. The metabolite was isolated by manually collecting the eluting peak from an HPLC run. The solvent was evaporated, and the remaining sample was analyzed by nuclear magnetic resonance spectroscopy (NMR) with an AV 400 spectrometer (Bruker, Fällanden, Switzerland).

Preparation of crude cell extract and enzyme assays. To prepare the crude cell extracts, strain 3-2W4 cells (≤20%, wt/vol) were suspended in 100 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.0) and disrupted by ultrasonication. The extracts were centrifuged for 15 min at 16,000 × *g* and 4°C. Unless otherwise stated, the enzyme assay mixtures contained 5 mM β-tripeptide, 100 mM MOPS buffer (pH 7.0), and BapA activity in limiting amounts. The reaction mixture was incubated at 30°C, and samples were withdrawn regularly, heated at 95°C for 3 min, and centrifuged. The supernatant was removed and analyzed by HPLC. One U is defined as the amount of enzyme that catalyzes the formation of 1 μmol β-dipeptide per minute. Protein was determined with a Bio-Rad protein assay (Bio-Rad, Reinach, Switzerland), with bovine serum albumin as the standard.

Protein purification. All protein purification steps were performed at 4°C. Three grams of cells of strain 3-2W4 was suspended in 9 ml of 50 mM Tris-HCl (pH 8.0) (buffer A), placed on ice, and disrupted by ultrasonication. The cell debris was separated by centrifugation, and the clear supernatant was loaded onto a Bio-Scale Q20 column (Bio-Rad, Reinach, Switzerland) equilibrated with buffer A. BapA was eluted with a linear gradient of sodium chloride (0 to 25 M) in buffer A. The active fractions were pooled and directly loaded onto a column packed with Phenyl Sepharose FF (low sub, 1.2 by 8.8 cm; Amersham Biosciences, Uppsala, Sweden) equilibrated with buffer A. The column was washed with buffer A, followed by a second washing step with 0.5 mM Tris-HCl (pH 8.0) (buffer B), and BapA was eluted with 50% (vol/vol) ethylene glycol in buffer B. The buffer of the active fractions was exchanged with buffer A, and the sample volume was reduced by ultrafiltration by means of a Centriprep-YM10 device (Millipore, Volketswil, Switzerland).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% polyacrylamide gels according to Lämmli (19), and the gels were stained with Coomassie brilliant blue G-250. Proteins were blotted on PVDF membranes with a Mini-Trans blot cell (Bio-Rad, Reinach, Switzerland), and the membranes were stained with Ponceau S.

N-terminal amino acid sequencing. The N-terminal amino acid sequences of the proteins were determined by automated Edman degradation with a Procise cLC protein sequencing system (Applied Biosystems, Rotkreuz, Switzerland).

DNA techniques and sequence analysis. Genomic DNA was isolated using an AquaPure genomic DNA isolation kit (Bio-Rad, Reinach, Switzerland). The following degenerated primers were designed based on the N-terminal sequence information of the purified enzyme and applied in PCR experiments using genomic DNA as the template to isolate a part of the *bapA* gene: for_35 (5′-TTCGARCCGACSCCSGGCGC-3′) and rev_36 (5′-GCRTCSGTSGCGATSACGAT-3′). The amplification procedure consisted of an initial denaturation step for 2 min at 95°C, followed by 6 cycles of 30 s at 95°C, 30 s at 60 to 50°C, and 1 min at 72°C and 25 cycles of 30 s at 95°C, 30 s at 54°C, and 1 min at 72°C. The reaction was stopped after a final extension for 5 min at 72°C. All PCRs were performed using *Taq* polymerase (Sigma, Buchs, Switzerland) with the supplied buffer. The complete *bapA* gene sequences were isolated with a Universal GenomeWalker kit (BD Biosciences, Basel, Switzerland) (17). Genomic DNA was cut with PvuII, EcoRV, StuI, and NruI. The GenomeWalker adaptors were ligated to the genomic DNA fragments, and these libraries were used as templates in nested PCRs with the following primers: Na_37 (5′-GGTGCCGTTGATCACGGCACGGCC-3′), Ni_38 (5′-CTGGTTTTCCCGAGCGGATGAATGAT-3′), Ca_39 (5′-GTCGTTTCGGCGGGGGACACGCG-3′), and Ci_40 (5′-AACCACGGCGACCGCAACGACCTGCGCAT-3′). DNA sequencing was carried out by Sequiserve (Vaterstetten, Germany) with an ABI Prism 3730 sequencer.

Nucleotide sequence accession numbers. The *bapA* and the partial 16S rRNA nucleotide sequences were submitted to the GenBank database under accession numbers AY897555 and AY950663.

RESULTS

Isolation and characterization of strain 3-2W4. In a previous study, we reported the enrichment of mixed microbial cultures that were capable of degrading the β-tripeptide H-βhVal-βhAla-βhLeu-OH and the β-dipeptide H-βhAla-βhLeu-OH (28). From this consortium, which was enriched from the aeration tank of a wastewater treatment plant, we initiated further enrichment cycles. After nine growth cycles in TriMM1, cells were plated on TGY agar medium and incubated for up to 7 days at 25°C. Four phenotypically different colonies were selected from the plates and isolated by subsequent streaking on TGY agar medium. All 15 possible combinations of these four strains were inoculated in TriMM1 and DiMM1, respectively. Growth and degradation of both β-peptides were observed only in flasks which contained strain 3-2W4. This bacterium degraded more than 90% of the supplied β-tripeptide within 9 to 14 days and around 80% of the supplied β-dipeptide within 20 days. After 1 week of incubation on TGY agar, strain 3-2W4 formed circular, smooth, yellow colonies with a diameter of 1 to 2 mm. Cells were rod shaped, gram negative, 0.6 to 0.8 μm in width, and 1.5 to 2.5 μm in length. The strain was oxidase, catalase, and aminopeptidase positive. It was able to utilize a limited range of substrates as the sole carbon source: acetate, citrate, succinate, pyruvate, α-leucine, α-valine, α-alanine, β-homoleucine, β-homovaline, β-homoalanine, β-tripeptide, and β-dipeptide. It did not grow on glucose, arabinose, maltose, mannitol, mannose, gluconate, adipate, phenylacetate, glycerol, ethanol, or methanol. The strain formed H_2S and did not hydrolyze starch, Tween 80, DNA, gelatin, or esculin. Strain 3-2W4 grew in the presence of 10 μg/ml streptomycin, but all other antibiotics tested and an increased streptomycin concentration (50 μg/ml) inhibited growth in liquid media. The partial 16S rRNA gene sequence analysis showed the highest matches with *Sphingomonas suberifaciens*, *S. natatoria*, and *S. ursincola* (96%). Analysis of cellular fatty acids also resulted in a pattern typical for the genus *Sphingomonas*. Just recently, resistance against streptomycin was described as a characteristic typical of *Sphingomonas* strains (40). When unclassified bacteria were included in the 16S rRNA gene sequence analysis, the closest match was strain Y2 with 100% identity (GenBank accession number AB084247). Due to these results, we conclude that the strain 3-2W4 belongs to a hitherto unknown species of the family *Sphingomonadaceae*.

Identification of metabolites. When strain 3-2W4 was grown in TriMM1, two metabolites which appeared successively in the culture supernatant were observed. In the beginning of the cultivation, the appearance of the first metabolite was directly linked to the degradation of the β-tripeptide. This metabolite was easily identified as the β-dipeptide, because the HPLC retention time, UV visible spectrum, and HPLC–MS-MS spectrum were identical with the reference substance. The second metabolite appeared after around 5 days in the supernatant of TriMM1. For isolation and identification of this compound, strain 3-2W4 was cultivated for 11 days in TriMM1 supplemented with yeast extract, because the addition of yeast extract accelerated the growth and increased the final concentration of the second metabolite. NMR spectroscopy and HPLC–MS-MS measurements indicated that this metabolite was the N-terminal-acetylated β-dipeptide Ac-βhAla-βhLeu-OH. The compound was chemically synthesized, and the chromatographic and chemical-physical properties were compared to those of the second metabolite. This comparison confirmed the identity of the metabolite. Therefore, the synthesized N-terminal-acetylated β-dipeptide could also be used for quantification purposes.

TABLE 1. Yield coefficients, growth rates, and remaining carbon and nitrogen concentrations for strain 3-2W4

Parameter	Result after growth in:	
	TriMM2	DiMM2
Yield (g biomass/g substrate)	0.31	0.24
Growth rate (h^{-1})	0.009	0.006
Remaining carbon according to DOC measurements (% [wt/wt])	19	26
Remaining carbon incorporated in the N-acetylated β-dipeptide (% [wt/wt])	7.8	23
Remaining nitrogen incorporated in the N-acetylated β-dipeptide (% [wt/wt])	6.7	22
Remaining nitrogen in the form of NH_4^+ (% [wt/wt])	43	60

Growth on the β-tri- and the β-dipeptide. Growth experiments in slightly modified minimal media (TriMM2 and DiMM2) with the β-tripeptide and β-dipeptide revealed that strain 3-2W4 utilized both substrates as the sole carbon, energy, and nitrogen sources. Growth in TriMM2 was completed after about 15 days (Fig. 1A to C). The first metabolite, the β-dipeptide, emerged from the β-tripeptide and reached a maximum concentration of 1.8 mM after 7 days. The β-tripeptide and subsequently the β-dipeptide were completely degraded. The second metabolite, the N-acetylated β-dipeptide, was formed after 3 to 5 days and accumulated to a final concentration of 0.5 mM in the culture supernatant (Fig. 1A). This means that 10% (mol/mol) β-tripeptide was transformed into the N-acetylated β-dipeptide. DOC measurements showed that 20% of the initial DOC was still present in the culture supernatant and that only 80% of the DOC was transformed, presumably into biomass and CO_2 (Fig. 1B). When strain 3-2W4 was cultivated in DiMM2, the N-acetylated β-dipeptide also occurred as metabolite (Fig. 1D) and accumulated to a final concentration of 0.9 mM, which corresponds to 22% (mol/mol) of the initial β-dipeptide. In this case, 26% of the total available carbon was not utilized during growth (Fig. 1E). The increase of pH during both cultivations was due to the formation of ammonia (Fig. 1C and 1F). Nitrate and nitrite could not be detected during the incubations. Table 1 shows growth yield coefficients, growth rates, and remaining carbon and nitrogen concentrations for strain 3-2W4 on the β-tripeptide and on the β-dipeptide.

Growth on complex medium and evidence for β-peptide-degrading activity in crude cell extracts. Strain 3-2W4 was grown in complex medium (LB) to investigate if any β-peptide-

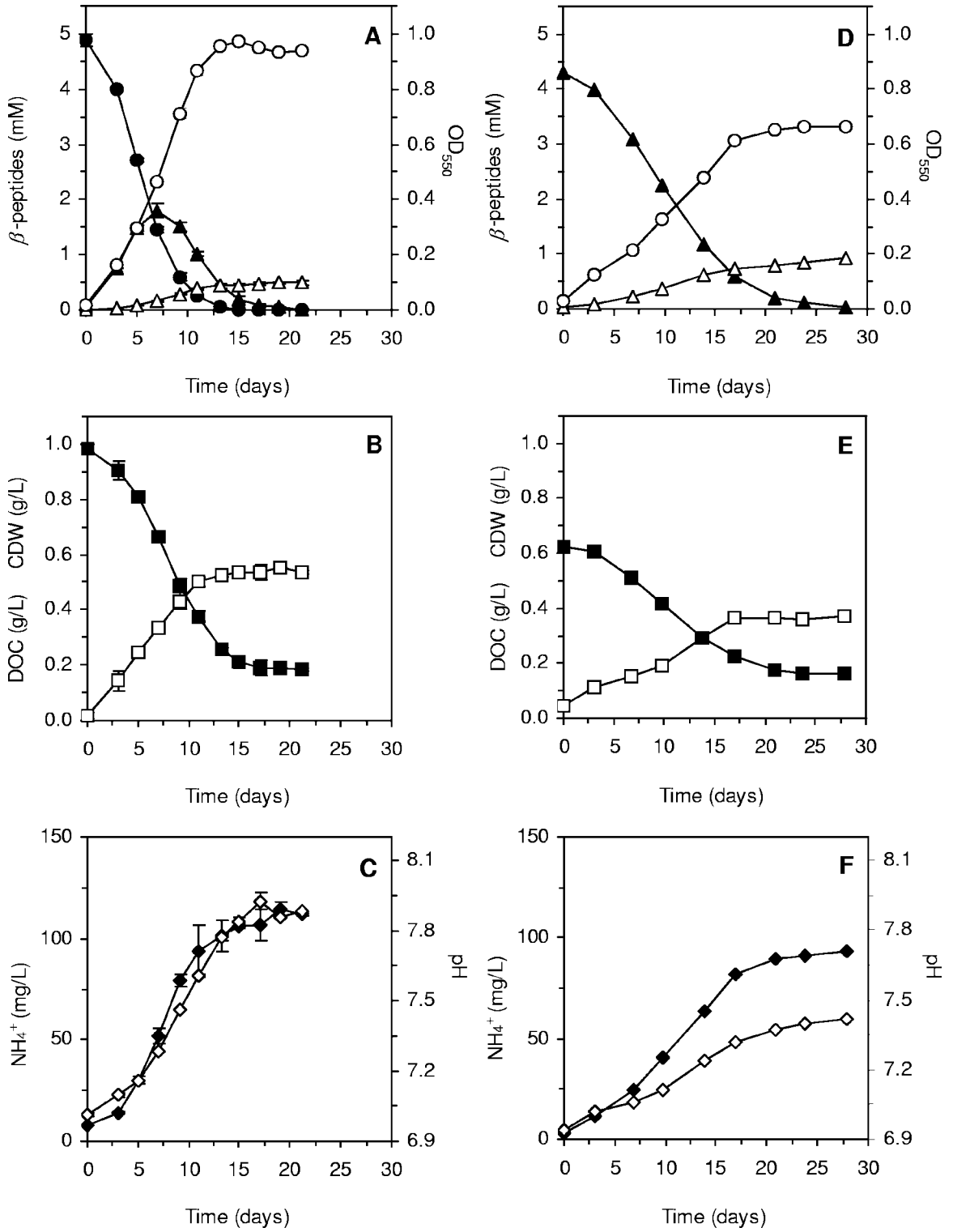

FIG. 1. Growth of strain 3-2W4 on β-tripeptide (A to C) and β-dipeptide (D to F). The following parameters were monitored over time for both growth substrates: β-tripeptide (●), β-dipeptide (▲), N-acetylated β-dipeptide (△), OD_{550} (○), DOC (■), cell dry weight (CDW) (□), pH (◇), and ammonia (◆). Two independent cultivations were performed for TriMM2, and one cultivation was performed for DiMM2 (due to limited availability of the substrate). Bars denote standard deviations.

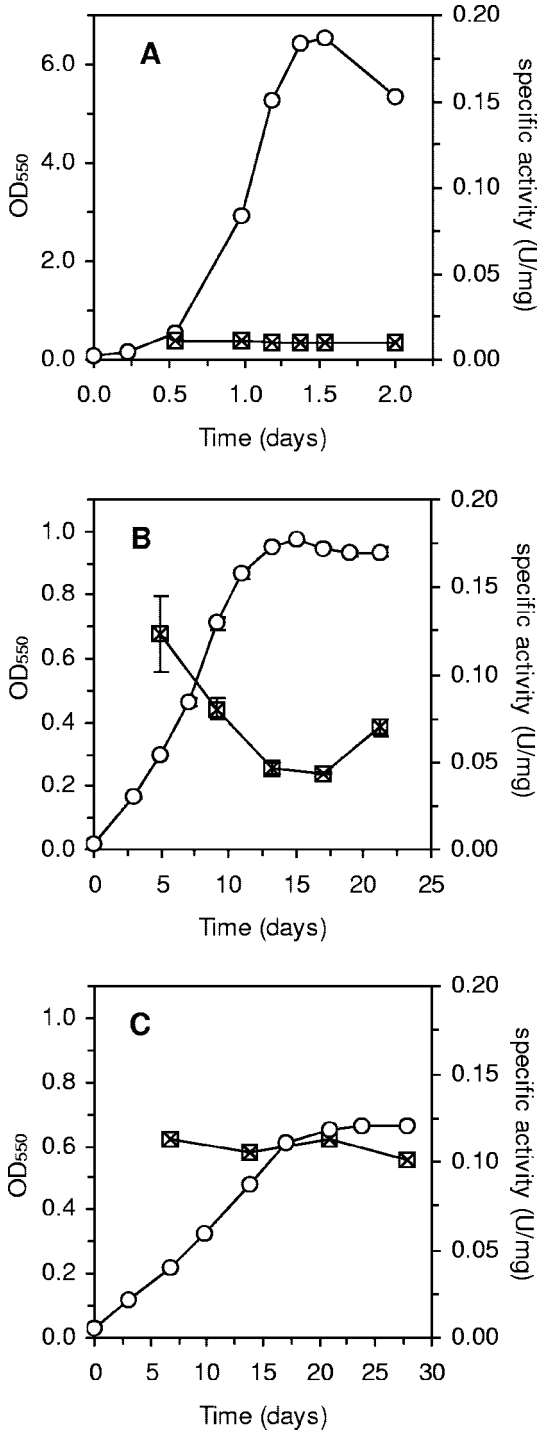

FIG. 2. Specific activities of BapA in the crude cell extracts of strain 3-2W4 cultivated in LB (A), TriMM2 (B), and DiMM2 (C). Symbols: ⊠, specific activity; ○, OD_{550}. Bars denote standard deviations.

degrading activity was induced under these conditions. Growth was completed after 1.5 days, the growth rate during the exponential phase was 0.15 h^{-1}, and the cells reached an optical density at 550 nm (OD_{550}) of 6.5 (Fig. 2A). β-Peptide-degrading enzyme activity was present in the crude cell extracts, and

TABLE 2. Purification scheme of BapA from strain 3-2W4[a]

Step	Total activity (U)	Total protein (mg)	Vol (ml)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude cell extract	3.0	160	10	0.019	1	100
MacroQ	2.15	76	35	0.028	1.5	71
Phenyl Sepharose	0.83	0.34	30	2.41	127	27

[a] The enzyme assay was performed in the presence of 0.1 M Tris-HCl buffer (pH 8.0) at 37°C.

we measured a specific activity of 0.010 U/mg of protein in all samples with the β-tripeptide as the substrate (Fig. 2A). The β-dipeptide was also degraded by strain 3-2W4 crude cell extract. When the cells were cultivated in TriMM1 and DiMM1, specific activities of 0.12 and 0.11 U/mg of protein, respectively, were reached (Fig. 2B and C). The β-peptides were degraded in the crude cell extract without the addition of any cofactors, cosubstrates, or metal ions. We concluded that this is a strong indication for the presence of a hydrolytic activity. No activating or inhibitory effects were observed when the enzyme assay was performed in the presence of 1 mM of EDTA, $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_2$, $MnCl_2$, $CuCl_2$, β-mercaptoethanol, 1,4-dithio-DL-threitol, or phenylmethylsulfonyl fluoride.

Purification and identification of the β-peptidyl aminopeptidase (BapA). In order to identify the β-peptide-degrading activity, a purification procedure was developed. The enzyme was purified by anion-exchange chromatography and hydrophobic-interaction chromatography, followed by an ultrafiltration step to exchange the buffer and to concentrate the sample. At this stage, the enzyme was purified 127-fold, with an overall yield of 27% (Table 2). The purified enzyme was capable of cleaving the nonnatural β-peptides H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH. Analysis of the enzyme preparation showed two major protein bands with molecular masses of 27 and 12 kDa by SDS-PAGE (Fig. 3). The N termini of both proteins were sequenced and compared to protein databases with a BLAST search for short, nearly exact matches (3). One of the closest matches for the N-terminal sequence of the 27-kDa protein (GPRARDLGVXFEGTPGA LNA) was the α-subunit of the L-aminopeptidase-D-amidase/D-esterase DmpA from *Ochrobactrum anthropi*. The β-subunit of the same enzyme was also among the sequences that produced significant alignments with the N terminus of the 12-kDa protein (SLLIVIATDAPLM). From these data, we concluded that the 27-kDa and the 12-kDa polypeptides form the active protein, and we named this novel enzyme β-peptidyl aminopeptidase (BapA).

Identification and characterization of the β-peptidyl aminopeptidase-encoding gene (*bapA*). Two degenerated primers (for_35 and rev_36) were designed on the basis of the N-terminal sequences from both subunits. With these primers, we obtained a PCR product, which had the expected size of approximately 700 bp, and sequenced it to confirm its identity to the *bapA* gene. The primary structure of the whole *bapA* gene, including flanking regions, was determined by applying a Universal GenomeWalker kit. Analysis of the sequence confirmed

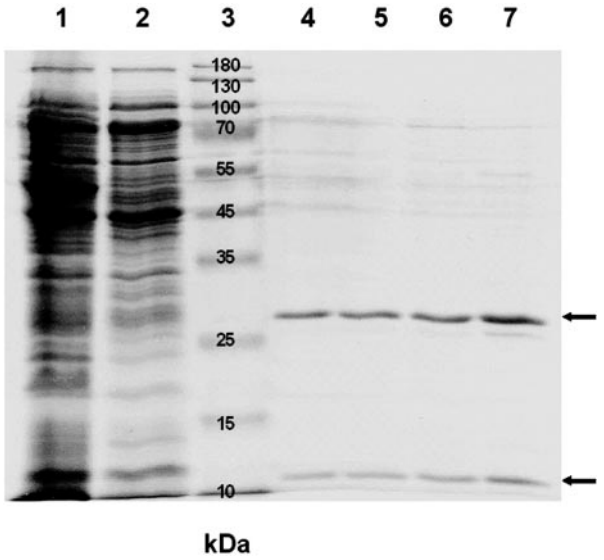

FIG. 3. SDS-PAGE analysis of samples containing BapA at different stages of purification from strain 3-2W4 cells. Lane 1, crude cell extract; lane 2, pool after anion-exchange chromatography; lane 3, marker; lanes 4 to 7, fractions 105 to 108, respectively, after hydrophobic-interaction chromatography. The arrows mark the two subunits of BapA.

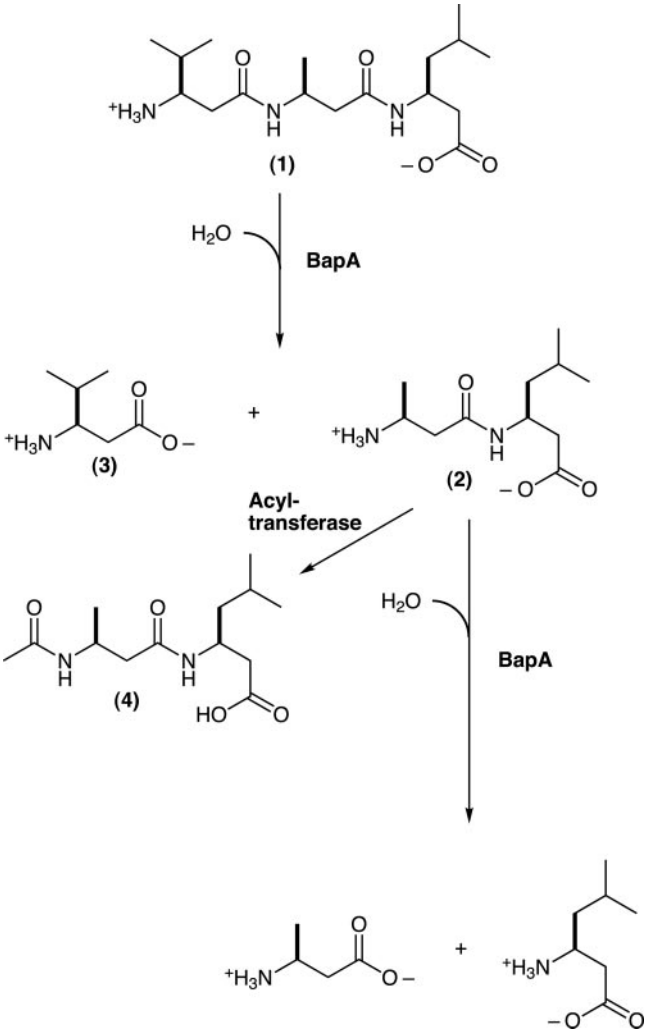

FIG. 4. Proposed degradation pathway of the β-tripeptide H-βhVal-βhAla-βhLeu-OH (no. 1). The β-peptidyl aminopeptidase BapA catalyzes the first degradation step of the β-tripeptide to the β-dipeptide H-βhAla-βhLeu-OH (no. 2) and βhVal (no. 3). The major part of the β-dipeptide was hydrolyzed by the same enzyme while a competing reaction led to the formation of the N-acetylated β-dipeptide Ac-βhAla-βhLeu-OH (no. 4).

that the sequence of the β-subunit was encoded in the *bapA* sequence directly after the presumed cleavage site of the encoded peptide. A potential ribosome binding site (AGGAA AGA) was found 7 nucleotides upstream of the putative initiation codon ATG at nucleotide 1. The GC content of the gene is 66.5%. The putative *bapA* gene encodes a preprotein of 402 amino acids corresponding to a theoretical mass of 41,531 Da. A comparison of the N-terminal amino acid sequence of the bigger subunit with the gene data showed that BapA carries a signal peptide of 29 amino acids. The presence of the signal sequence, the location of the cleavage site, and the subcellular localization were confirmed by the prediction results of the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) (6) and the TargetP V1.0 server (http://www.cbs.dtu.dk/services/TargetP/) (8). Assuming a cleavage of processed BapA into two subunits corresponding to residues 30 to 278 and 279 to 402, the two distinct polypeptides have molecular masses of 25.4 and 13.2 kDa, respectively. These values agree well with the protein bands of the purified enzyme preparation (Fig. 3).

DISCUSSION

Previously, we have described a bacterial enrichment culture that was able to metabolize nonnatural β-tri- and β-dipeptides (28). Here, we show that strain 3-2W4 was responsible for the degradation of these β-peptides. It is notable that strain Y2, the closest phylogenetic match to strain 3-2W4, is able to degrade microcystin, a cyclic peptide that has a high hepatic toxicity and contains β-peptidic structural elements (24, 27).

During degradation experiments with strain 3-2W4, both β-peptides, H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH, disappeared completely from the culture supernatant when they were applied as the sole carbon, energy, and nitrogen source. While the β-tripeptide was metabolized to the β-dipeptide, we observed two reactions for the further metabolism of H-βhAla-βhLeu-OH (Fig. 4). A major amount of H-βhAla-βhLeu-OH was completely metabolized, but a small part was transformed to a persisting metabolite that was subsequently identified as the N-terminal-acetylated β-dipeptide Ac-βhAla-βhLeu-OH. The growth rate and the growth yield coefficient of strain 3-2W4 were higher when cells grew on the β-tripeptide than when they grew on the β-dipeptide (Table 1). This observation is consistent with the presence of a higher concentration of N-acetylated β-dipeptide at the end of cultivations with the β-dipeptide medium.

Experiments with cell extracts showed that a hydrolytic β-peptide-degrading activity was present in crude cell extracts of β-peptide-grown cells of strain 3-2W4 (Fig. 2B and C). This activity was also present at a low, constant level when cells grew

in LB medium (Fig. 2A). The β-peptide-degrading activity was highly enriched (Table 2), and SDS-PAGE analysis indicated the presence of two subunits with molecular masses of 27 and 12 kDa, respectively (Fig. 3). The N termini of both subunits were sequenced, and the sequences were compared to those of protein databases. DmpA, an aminopeptidase from *O. anthropi* (23), closely matched the sequences of both subunits. From our transformation data and from the results of the sequence analysis, we concluded that the purified β-peptide-hydrolyzing activity is due to a β-peptidyl aminopeptidase. This conclusion was further supported by the isolation and sequence analysis of the gene that encodes the enzyme. Therefore, we named the enzyme BapA for β-peptidyl aminopeptidase.

DmpA from *O. anthropi* is described as the prototype of a new family of Ntn hydrolases (23), which are activated by a self-catalyzed protein splicing process between two conserved residues to open access to the catalytic N-terminal residue (serine, threonine, or cysteine) (9). The two subunits of DmpA from *O. anthropi* have molecular masses of 26.6 and 13.7 kDa, respectively; this corresponds well with the molecular masses of the BapA subunits. Forty-two percent of the deduced amino acid sequence of BapA from strain 3-2W4 was identical to that of DmpA from *O. anthropi* (total alignment length, 372 amino acids) (data not shown). These two structurally related enzymes catalyze the hydrolysis of amide bonds in nonnatural oligopeptides, but the exact substrate specificities need to be evaluated yet. The results of our inhibition studies revealed that BapA was inhibited neither by the protease inhibitors phenylmethylsulfonyl fluoride and EDTA nor by divalent metal ions and reducing agents. No inhibitor has been found so far for DmpA from *O. anthropi* (9). BapA remains active even in the presence of 50% (vol/vol) ethylene glycol during purification. The presence of disulfide bridges can be excluded because of the absence of cysteines. The cleavage site of BapA (N278 to G279) is similar to that found in other Ntn hydrolases (9) but different from that of DmpA. While DmpA from *O. anthropi* is located in the cytoplasm, BapA contains a signal sequence of 29 amino acids indicating a possible translocation into the periplasm. The presence of a signal sequence is a common feature within several other families of Ntn hydrolases (22, 36, 37). The putative periplasmic location of BapA might be a prerequisite for the utilization of the β-peptides H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH by strain 3-2W4, because it might eliminate the need for a suitable β-peptide transport system.

Many prior studies showed that β-peptides are extremely stable against diverse proteases and peptidases (10, 14–16, 20, 29, 32). Here we describe for the first time an enzyme that is able to cleave β-peptide bonds of nonnatural β-peptides. The periplasmic β-peptidyl aminopeptidase BapA from strain 3-2W4 belongs to a new family of Ntn hydrolases and plays a crucial role in the metabolism of such peptides by strain 3-2W4. Further work on the substrate spectrum and the kinetic properties of this unusual enzyme is on the way.

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