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Bacterial β-peptidyl aminopeptidases with unique substrate specificities for β-oligopeptides and mixed β,α-oligopeptides

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Database
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We previously discovered that BapA, a bacterial β-peptidyl aminopeptidase, is able to hydrolyze two otherwise metabolically inert β-peptides [Geueke B, Namoto K, Seebach D & Kohler H-PE (2005) *J Bacteriol* **187**, 5910–5917]. Here, we describe the purification and characterization of two distinct bacterial β-peptidyl aminopeptidases that originated from different environmental isolates. Both *bapA* genes encode a preprotein with a signal sequence and were flanked by ORFs that code for enzymes with similar predicted functions. To form the active enzymes, which had an $(\alpha\beta)_4$ quaternary structure, the preproteins needed to be cleaved into two subunits. The two β-peptidyl aminopeptidases had 86% amino acid sequence identity, hydrolyzed a variety of β-peptides and mixed β/α-peptides, and exhibited unique substrate specificities. The prerequisite for peptides being accepted as substrates was the presence of a β-amino acid at the *N*-terminus; peptide substrates with an *N*-terminal α-amino acid were not hydrolyzed at all. Both enzymes cleaved the peptide bond between the *N*-terminal β-amino acid and the amino acid at the second position of tripeptidic substrates of the general structure H-βhXaa-Ile-βhTyr-OH according to the following preferences with regard to the side chain of the *N*-terminal β-amino acid: aliphatic and aromatic > OH-containing > hydrogen, basic and polar. Experiments with the tripeptides H-D-βhVal-Ile-βhTyr-OH and H-βhVal-Ile-βhTyr-OH demonstrated that the two BapA enzymes preferred the peptide with the L-configuration of the *N*-terminal β-homovaline residue as a substrate.

β-Peptides consisting of β-amino acids carrying side chains of the 20 proteinogenic α-amino acids were synthesized for the first time in 1996 [1] and have been intensively studied ever since [2]. This new class of compounds exhibits unexpected properties, such as high metabolic stability [3] and the ability to adopt stable secondary structures [4,5] and mimic cationic cell-penetrating peptides [6,7]. β-Peptides have been reported to be extraordinarily resistant against degradation by many common peptidases and proteases [1,8–13]. Because of these properties, β-peptides are considered to be pharmaceutically interesting agents that act as peptidomimetics and specific inhibitors [14,15]. Natural peptides solely composed of β-amino acids are not known so far, but β-amino acid structures do occur in mixed peptides such as carnosine, bestatin, and microcystin, and in various other biological molecules, such as pantothenic acid, cocaine, and paclitaxel.

Abbreviations
DmpA, L-aminopeptidase-D-amidase/D-esterase; *p*NA, *p*-nitroanilide; Ps BapA, β-Ala-Xaa dipeptidase from *Pseudomonas* sp. MCI3434; 3-2W4 BapA, β-peptidyl aminopeptidase from strain 3-2W4; Y2 BapA, β-peptidyl aminopeptidase from strain Y2.

Enrichment studies with mixed microbial cultures gave the first evidence of the biodegradability of β-peptides [16]. One bacterial isolate, designated strain 3-2W4, was able to grow on two specific β-peptides (H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH; nomenclature according to [2]) and to degrade them completely [17]. Strain 3-2W4 was assigned to the newly described genus *Sphingosinicella*, and was recently named *Sphingosinicella xenopeptidilytica* 3-2W4 [18]. The closest phylogenetic match is *S. microcystinivorans* Y2, which was isolated from a freshwater lake in Japan and is capable of degrading microcystin, a cyclic, toxic heptapeptide that contains β-peptidic substructures [19–21].

The degradation of the two β-peptides H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH, is catalyzed by a novel β-peptidyl aminopeptidase that was named BapA [17]. The deduced amino acid sequence of the enzyme is similar to that of the L-aminopeptidase-D-amidase/D-esterase DmpA from *Ochrobactrum anthropi* LMG7991 [22] and that of the β-Ala-Xaa-dipeptidase BapA from *Pseudomonas* sp. MCI3434 [23]. These related enzymes exhibit unusual peptidase, esterase and amidase specificities. DmpA from *O. anthropi* hydrolyzes the chromogenic substrate H-D-Ala-*p*NA and short α-peptides composed of L-amino acids with good efficiencies, whereas BapA from *Pseudomonas* sp. does not cleave α-peptides, but peptides and amides with βhGly (also commonly named βAla) at the *N*-terminal position. Analysis of the purified proteins and the gene sequences indicated that DmpA, BapA from *Pseudomonas* sp. and BapA from strain 3-2W4 are translated as preproteins and cleaved into two subunits at a conserved site in front of a serine [17,22,23]. For DmpA, mutagenesis studies suggested that this serine is essential for both enzymatic activity and cleavage of the preprotein [22]. These enzymes constitute a novel group of aminopeptidases with unusual activities for short peptides composed of nonproteinogenic amino acids [22,23], and they play a key role in the biodegradation of non-natural β-peptides [17]. Here, we report the cloning, genetic analysis and biochemical characterization of two β-peptidyl aminopeptidases. The enzymes have similar, but exceptional, substrate specificities. They hydrolyze a variety of β-oligopeptides and mixed β/α-oligopeptides, but do not accept α-amino acids at the *N*-terminal position of the substrate peptides.

Results

β-Peptide utilization by strain Y2

Growth experiments in minimal media with the β-tripeptide H-βhVal-βhAla-βhLeu-OH and the β-dipeptide H-βhAla-βhLeu-OH revealed that strain Y2, like strain 3-2W4, was able to utilize both β-peptides as the sole carbon, energy and nitrogen sources. Under the experimental conditions, the β-dipeptide was completely degraded after 14 days, and the β-tripeptide after 31 days. Small amounts of the *N*-acetylated β-dipeptide Ac-βhAla-βhLeu-OH were formed during growth on both substrates.

Genetic analysis of the *bapA* genes from strains Y2 and 3-2W4

We designed PCR experiments with degenerated primers (for_35 and rev_36) to screen the genomic DNA of strain Y2 for a gene sequence similar to *bapA* from strain 3-2W4. A PCR product with the expected size of 700 bp was obtained and sequenced. The flanking regions of this novel sequence were determined by genome walking, and one ORF that encodes a protein of 399 amino acids was identified. A potential ribosome-binding site (AGGGAAGG) was found seven nucleotides upstream of the start codon. The gene sequence was compared to protein databases with a translating BLASTX search [24]. The closest match was the β-peptidyl aminopeptidase BapA from strain 3-2W4 (86% amino acid identity). Therefore, the gene was also named *bapA*. We found two further, functionally characterized proteins among the sequences that produced significant alignments: the β-Ala-Xaa dipeptidase BapA from *Pseudomonas* sp. (35% amino acid identity to Y2 BapA) and the L-aminopeptidase-D-amidase/D-esterase DmpA from *O. anthropi* (39% amino acid identity to Y2 BapA). The sequences of these four proteins were aligned and are shown in Fig. 1. Comparison of the *N*-terminal amino acid sequences of the aligned proteins indicated that Y2 BapA, like 3-2W4 BapA, carries a signal peptide. This observation was supported by the predictions of the SIGNALP 3.0 [25] and TARGETP V1.0 servers [26], as well as the presence of net positive charges in the N regions of the signal peptides, the presence of glycyl or prolyl residues that could function as a helix breaker in the H domains, and the presence of typical residues with small neutral side chains located at positions −1 and −3 relative to the start of the mature proteins [17,27].

The flanking regions of the *bapA* genes from strains 3-2W4 and Y2 were sequenced and analyzed by BLAST searches. Upstream and downstream of both *bapA* genes, partial ORFs were identified that code for putative sugar transporters and threonine dehydratases, respectively.

Genomic DNA of strain 3-2W4 was digested with endonucleases that did not cut within the *bapA* ORF

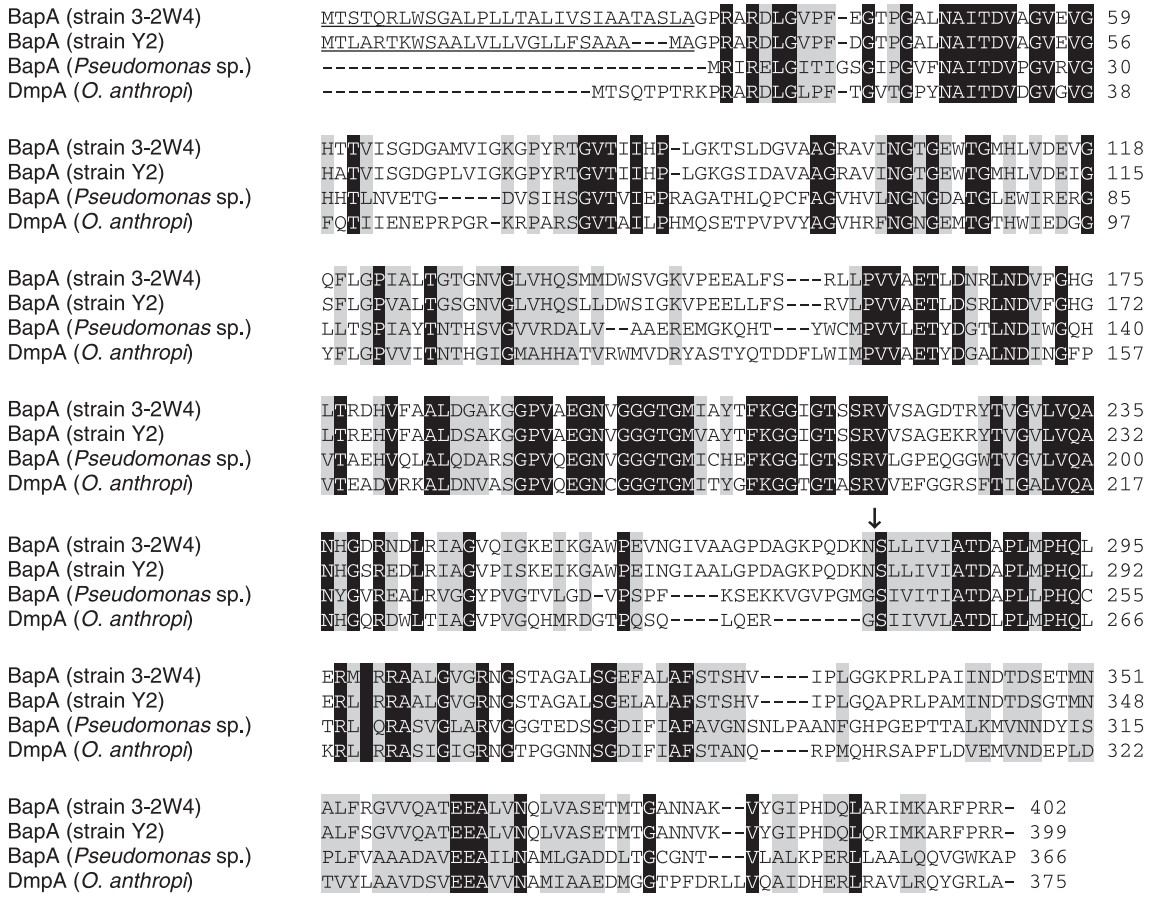

Fig. 1. Alignment of the amino acid sequences of BapA from strain 3-2W4, *Pseudomonas* sp. strain Y2 and DmpA from *Ochrobactrum anthropi*. Identical amino acids are marked in black and similar amino acids are marked in gray. The cleavage sites of the proteins are marked with an arrow. The signal sequences of BapA from strains 3-2W4 (residues 1–29) and Y2 (residues 1–26) are underlined. The sequences have the following accession numbers: BapA from strain 3-2W4, AAX93858; BapA from strain Y2, DQ323513; BapA from *Pseudomonas* sp. MCI3434, BAE02664; DmpA from *O. anthropi* LMG7991, CAA66259.

(*Hind*III, *Sac*I, *Eco*RV, *Pst*I, *Nco*I). On a Southern blot, single bands were obtained when the DNA was probed with 640 bp of the *bapA* gene. This revealed that the *bapA* gene is present as a single copy in the genome of strain 3-2W4.

Production and purification of 3-2W4 BapA and Y2 BapA

The *bapA* sequences from strains 3-2W4 and Y2 were amplified by PCR and cloned into the expression vector pET3c. The 5′-termini of both genes corresponding to the putative signal sequences were omitted, and additional start codons were introduced to ensure a cytoplasmic location of the enzymes in *Escherichia coli* BL21(DE3) pLysS. Both recombinant strains harboring the plasmids p3BapA and pYBapA, respectively, were cultivated in fed-batch fermentations. With these high-cell-density cultivations, A_{450} values of 111 and 107 were reached, yielding 320 g and 305 g of cells (wet weight), respectively. The biosynthesis of the recombinant enzymes was verified by SDS/PAGE analysis and enzyme activity measurements. The two enzymes were purified in a two-step chromatography procedure (Table 1). Total activities of 1.9 U and 2.3 U for 3-2W4 BapA and Y2 BapA, respectively, were obtained from 2 g of cells (wet weight). SDS/PAGE analysis of the purified proteins revealed that Y2 BapA was composed of two subunits with similar molecular masses to those of the two subunits of 3-2W4 BapA (Fig. 2). The purities of 3-2W4 BapA and Y2 BapA were 98% and 96%, respectively, according to digital image analysis of the polyacrylamide gels (Fig. 2). The increase in yields after the first chromatography step might be caused by elimination of inhibitors and competing substrates present in crude

Table 1. Purification schemes of recombinant 3-2W4 BapA and Y2 BapA. The enzyme activity was assayed by following the hydrolysis of 5 mM H-βhGly-*p*NA at 25 °C in the presence of 50 mM Tris/HCl (pH 8.0), 10% (v/v) dimethylsulfoxide, and enzyme in limiting amounts. One unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmol of *p*-nitroaniline per minute.

	3-2W4 BapA						Y2 BapA					
	Total activity (U)	Total protein (mg)	Activity ($U \cdot mL^{-1}$)	Specific activity ($U \cdot mL^{-1}$)	Purification (fold)	Yield (%)	Total activity (U)	Total protein (mg)	Activity ($U \cdot mL^{-1}$)	Specific activity ($U \cdot mL^{-1}$)	Purification (fold)	Yield (%)
Crude extract	4.8	224	0.24	0.021	1	100	2.3	350	0.11	0.0064	1	100
MacroQ	7.4	91	0.26	0.081	4	154	5.3	99	0.21	0.054	8	236
Phenyl Sepharose	4.4	4.2	0.20	1.02	48	91	4.2	14.4	0.21	0.29	45	185
Lyophilization[a]	1.9	3.1	0.09	0.62	29	40	2.3	11.5	0.12	0.20	31	103

[a] For activity and protein analysis, an aliquot of the lyophilized protein was redissolved in 50 mM Tris/HCl (pH 8.0).

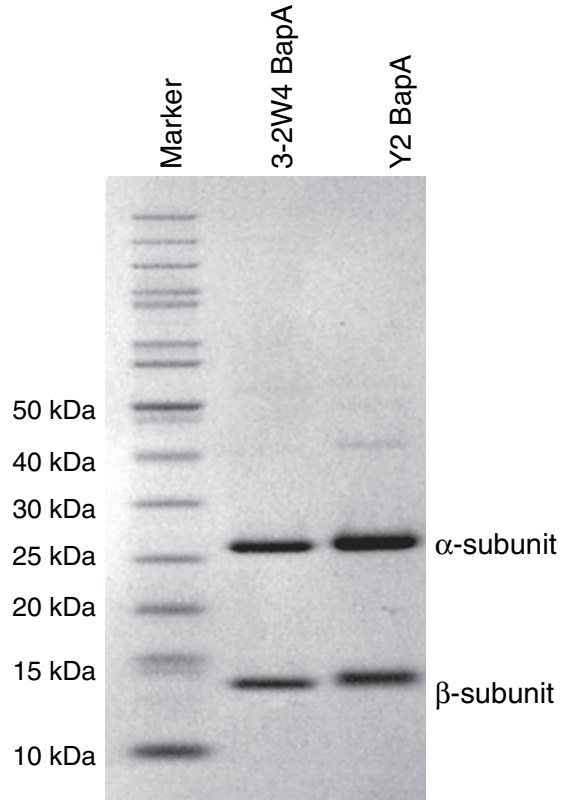

Fig. 2. SDS/PAGE analysis of the purified enzymes 3-2W4 BapA and Y2 BapA. According to the gel, the molecular masses of the α-subunits and β-subunits were 26.6 kDa and 13.4 kDa, respectively, for 3-2W4 BapA, and 26.8 kDa and 13.9 kDa, respectively, for Y2 BapA.

extracts or by the continuing cleavage of the preprotein during the purification. After purification and lyophilization, the specific activities were 0.62 and 0.20 $U \cdot mg^{-1}$ for 3-2W4 BapA and Y2 BapA, respectively, as measured under standard assay conditions with the chromogenic, commercially available substrate H-βhGly-*p*NA.

Table 2. Comparison of substrate specificities of 3-2W4 BapA and Y2 BapA. The values represent one experiment. ND, not detectable. The specific activities of the two BapA enzymes for the first five peptides were quantified by HPLC of the residual substrate; the reactions of the other substrates were analyzed by measuring the formation of the dipeptide H-Ile-βhTyr-OH. The starting concentration of all substrates was 2.5 mM.

	Specific activity	
Substrate	3-2W4 BapA ($U \cdot mg^{-1}$)	Y2 BapA ($U \cdot mg^{-1}$)
H-βhVal-βhAla-βhLeu-OH	3.1	0.84
H-βhAla-βhLeu-OH	1.1	3.1
H-Val-Ala-Leu-OH	ND	ND
Carnosine[a]	0.026	0.063
Bestatin	ND	ND
H-βhGly-Ile-βhTyr-OH	0.009	0.047
H-βhVal-Ile-βhTyr-OH	0.98	0.45
H-βhLeu-Ile-βhTyr-OH	1.9	0.38
H-βhPhe-Ile-βhTyr-OH[b]	0.68	0.46
H-βhTyr-Ile-βhTyr-OH	0.47	0.21
H-βhTrp-Ile-βhTyr-OH[b]	0.047	0.040
H-βhSer-Ile-βhTyr-OH	0.095	0.40
H-βhThr-Ile-βhTyr-OH	0.068	0.050
H-βhGln-Ile-βhTyr-OH	0.007	0.008
H-βhPro-Ile-βhTyr-OH	ND	ND
H-βhHis-Ile-βhTyr-OH	0.008	0.011
H-βhLys-Ile-βhTyr-OH	0.017	0.015
H-βhArg-Ile-βhTyr-OH	0.006	0.011
H-βhGlu-Ile-βhTyr-OH	ND	< 0.001
H-D-βhVal-Ile-βhTyr-OH	0.028	0.016

[a] The assay mixture contained 0% dimethylsulfoxide. [b] The assay mixture contained 40% dimethylsulfoxide.

Kinetic properties and substrate specificities of the two β-peptidyl aminopeptidases

Both enzymes hydrolyzed the two β-peptides H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH with high activities (Table 2). For H-βhVal-βhAla-βhLeu-OH, the only detected peptidic intermediate was

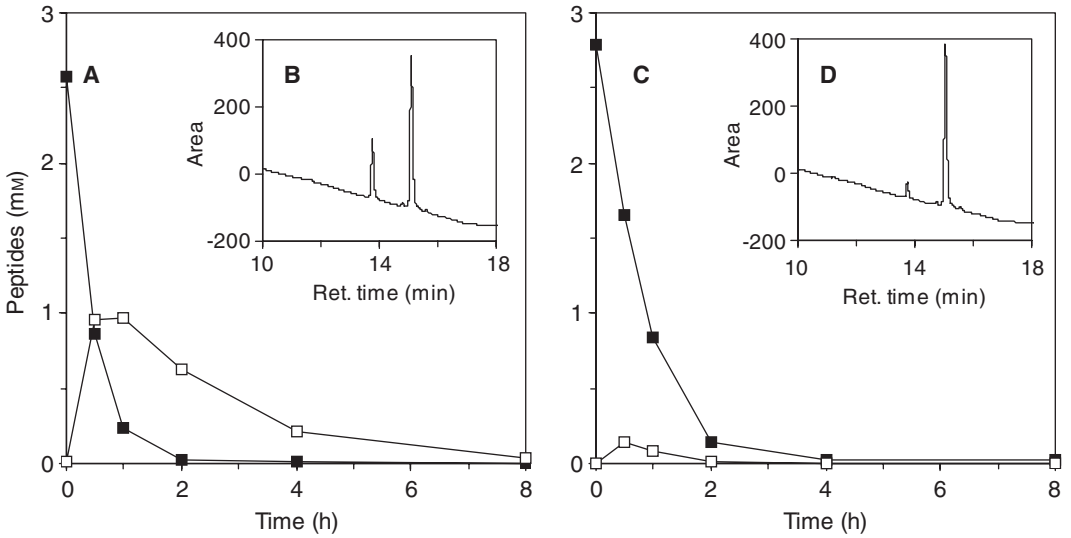

Fig. 3. Hydrolysis of H-βhVal-βhAla-βhLeu-OH by 15 μg·mL^{-1} 3-2W4 BapA (A) and 45 μg·mL^{-1} Y2 BapA (C). The inserts (B, D) illustrate the HPLC profiles after a reaction time of 30 min. The substrate H-βhVal-βhAla-βhLeu-OH (■) and the intermediate H-βhAla-βhLeu-OH (□) were identified by comparison with the reference substances and MS (H-βhVal-βhAla-βhLeu-OH, retention time = 15.1 min, $[M + H]^+$ 344.5; H-βhAla-βhLeu-OH, retention time = 13.8 min, $[M + H]^+$ 231.4).

H-βhAla-βhLeu-OH (Fig. 3). The α-tripeptide H-Val-Ala-Leu-OH and bestatin were not accepted as substrates, whereas carnosine was cleaved with low activity (Table 2). No degradation of DL-pyroglutamic acid-*p*NA was detected when this substrate was assayed spectrophotometrically under standard conditions with 3-2W4 BapA and Y2 BapA.

The kinetic parameters of 3-2W4 BapA and Y2 BapA were determined for H-βhVal-βhAla-βhLeu-OH, H-βhAla-βhLeu-OH, carnosine and H-βhGly-*p*NA (Table 3). 3-2W4 BapA cleaved the β-peptides H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH with high activities, whereas Y2 BapA hydrolyzed H-βhAla-βhLeu-OH faster than H-βhVal-βhAla-βhLeu-OH. The *N*-terminal βhGly was released slowly from carnosine and H-βhGly-*p*NA by both enzymes.

To elucidate the importance of the relative positions of α-amino acids and β-amino acids in such peptide substrates with regard to their enzymatic cleavage, a series of eight tripeptides of the general sequence valine, isoleucine and tyrosine with all possible combinations of α-amino acids and β-homoamino acids was synthesized. The two BapA enzymes hydrolyzed all peptides that had a β-homoamino acid at the *N*-terminal position (Fig. 4), but none of the peptides with an *N*-terminal α-amino acid. When the two mixed β/α-tripeptides H-βhVal-Ile-βhTyr-OH and H-βhVal-Ile-Tyr-OH, both of which contain an α-amino acid at the

Table 3. Kinetic constants of 3-2W4 BapA and Y2 BapA for different β-homoamino acid-containing peptides and H-βhGly-*p*NA. The release of the *N*-terminal β-homoamino acid of the peptides was measured at 37 °C and analyzed by HPLC. The formation of *p*-nitroaniline from H-βhGly-*p*NA was measured spectrophotometrically at 405 nm and 25 °C. The values are the average of three replicates and the errors represent the standard deviations.

	3-2W4 BapA			Y2 BapA		
Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}·s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}·s^{-1}$)
H-βhVal-βhAla-βhLeu-OH	9.0 ± 0.8	6.0 ± 0.2	670 ± 70	39 ± 15	4.3 ± 1.1	110 ± 50
H-βhAla-βhLeu-OH	20 ± 7	12 ± 2	590 ± 240	41 ± 16	60 ± 13	1500 ± 600
Carnosine[a]			8.5 ± 0.2			17 ± 3
H-βhGly-*p*NA	8.2 ± 1.9	0.75 ± 0.07	92 ± 23	4.4 ± 1.9	0.13 ± 0.01	29 ± 8

[a] The enzymes' velocities showed a linear dependency on the carnosine concentration (0–50 mM). The k_{cat}/K_m values were calculated according to the equation $k_{cat}/K_m = v/([E_0]·[S])$, where v is the velocity of the reaction, $[E_0]$ the stoichiometric concentration of active centers and [S] the carnosine concentration.

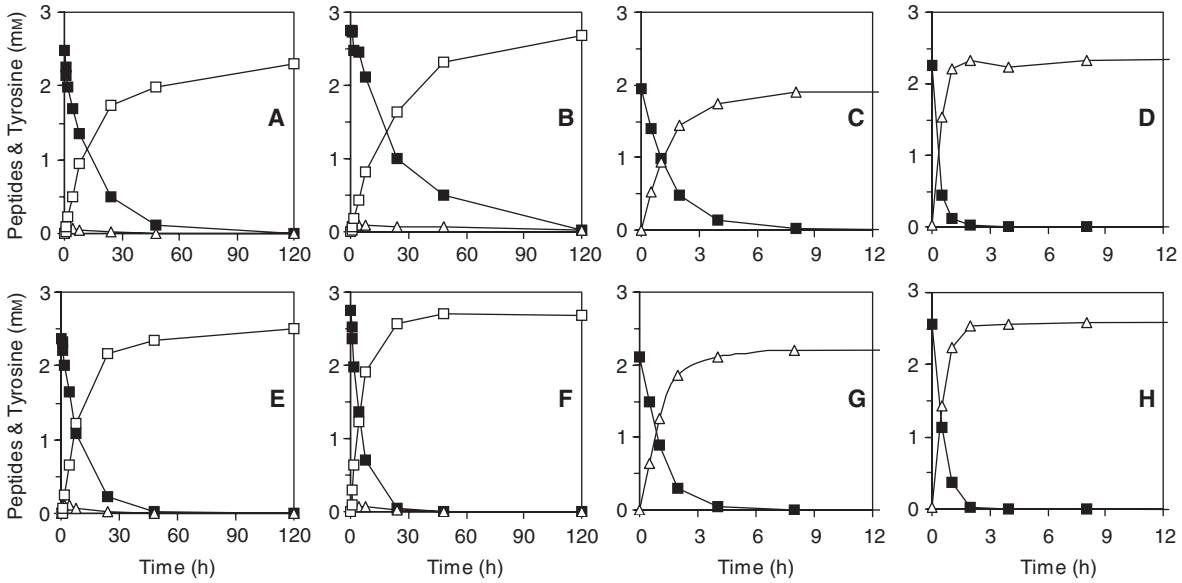

Fig. 4. Members of a series of eight tripeptides of the general sequence valine, isoleucine and tyrosine with all possible combinations of α-amino acids and β-homoamino acids were used as substrates for 3-2W4 BapA (A–D) and Y2 BapA (E–H). No degradation was observed for the substrates H-Val-Ile-Tyr-OH, H-Val-Ile-βhTyr-OH, H-Val-βhIle-Tyr-OH, and H-Val-βhIle-βhTyr-OH; the corresponding graphs are not shown. The assay mixtures contained 15 and 45 $\mu g \cdot mL^{-1}$, respectively, of 3-2W4 BapA and Y2 BapA. (A, E) ■, H-βhVal-βhIle-βhTyr-OH; △, H-βhIle-βhTyr-OH; □, βhTyr. (B, F) ■, H-βhVal-βhIle-Tyr-OH; △, H-βhIle-Tyr-OH; □, Tyr. (C, G) ■, H-βhVal-Ile-βhTyr-OH; △, H-Ile-βhTyr-OH. (D, H) ■, H-βhVal-Ile-Tyr-OH; △, H-Ile-Tyr-OH.

second position, were used as substrates, only the *N*-terminal βhVal was released and the remaining dipeptides were not cleaved. However, the tripeptides H-βhVal-βhIle-βhTyr-OH and H-βhVal-βhIle-Tyr-OH were completely degraded, and during the reaction only very low amounts of the intermediate dipeptides H-βhIle-βhTyr-OH and H-βhIle-Tyr-OH accumulated. These results show clearly that in order to be a substrate, a peptide requires an *N*-terminal β-homoamino acid.

β/α-Tripeptides with the general sequence H-βhXaa-Ile-βhTyr-OH, in which the *N*-terminal β-amino acid was varied systematically, were synthesized to gain information about which *N*-terminal β-amino acids were preferentially split off. The molecules were designed with the α-amino acid Ile at the second position so that only the variable *N*-terminal β-amino acid was split off and the remaining dipeptide H-Ile-βhTyr-OH was not further hydrolyzed. The resulting 15 peptides were incubated with 3-2W4 BapA and Y2 BapA (Table 2). The two enzymes had similar substrate specificities, with high activities for peptides with an *N*-terminal βhVal, βhLeu, βhPhe and βhTyr and rather low activities for peptides with a positively charged or polar β-homoamino acid at the *N*-terminus (βhArg, βhLys, βhGln). Generally, the specific activities of 3-2W4 BapA were higher than those of Y2 BapA, but the latter cleaved H-βhSer-Ile-βhTyr-OH, H-βhGly-Ile-βhTyr-OH and carnosine faster than did 3-2W4 BapA. Tripeptides with βhGlu and βhPro at the *N*-terminal position were not hydrolyzed at all by the two enzymes. Both enzymes showed selectivity with respect to the peptides H-βhVal-Ile-βhTyr-OH and H-D-βhVal-Ile-βhTyr-OH. The rates of cleavage of D-βhVal by 3-2W4 BapA and Y2 BapA were slower by factors of 35 and 28, respectively, as compared to the L-enantiomer (Table 2). 3-2W4 BapA and Y2 BapA hydrolyzed the chromogenic substrate H-D-Ala-*p*NA with rather low specific activities of 0.002 $U \cdot mg^{-1}$ and 0.007 $U \cdot mg^{-1}$, respectively. Neither 3-2W4 BapA nor Y2 BapA cleaved H-Ala-*p*NA.

Inhibitor studies

The inhibitory effects of various compounds on the hydrolysis of the β-tripeptide H-βhVal-βhAla-βhLeu-OH by 3-2W4 BapA and Y2 BapA were investigated. Under the tested conditions, both enzymes were completely inhibited by Pefabloc SC (0.4 and 4 mM), but not inhibited in the presence of EDTA (0.1, 1 and 10 mM), leupeptin (0.01, 0.1 and 1 mM), phenylmethanesulfonyl

fluoride (1 and 10 mM), bestatin (0.01 and 0.1 mM), and 1,10-phenanthroline (1 and 10 mM).

Influence of pH and temperature

The pH dependency was measured under standard assay conditions in the presence of a universal buffer (pH 4–11) [28]. 3-2W4 BapA exhibited maximal activities at pH values between 8 and 9, whereas Y2 BapA had a slightly narrower pH optimum, with a maximum at 10. No activity loss was observed when 3-2W4 BapA was incubated at 60 °C for 24 h. At 70 °C, the half-life of the enzyme was about 26 min. Y2 BapA had a half-life of approximately 1 h at 60 °C, and was completely inactivated after 5 min of incubation at 70 °C.

Molecular masses

BapA from strain Y2 was submitted to MALDI-TOF MS, and two peptides with molecular masses of 25 465 Da and 13 168 Da were identified. These values agree with the theoretical molecular masses of 25 332.7 Da and 13 144.1 Da that were calculated assuming a cleavage of the Y2 BapA preprotein between the conserved residues N275 and S276.

The native molecular masses of 3-2W4 BapA and Y2 BapA were determined by size exclusion chromatography and ESI MS. According to size exclusion chromatography, the native molecular masses of both enzymes were about 130 kDa. However, they coeluted with the DmpA from *O. anthropi*, which has a native molecular mass of 161 kDa [29]. This shows that size exclusion chromatography slightly underestimated the native molecular mass of these proteins. Therefore, we also performed a molecular mass determination by ESI MS under native conditions. This experiment yielded molecular masses of 150 230 Da and 155 805 Da for native 3-2W4 BapA and Y2 BapA, respectively. From these data, we conclude that both enzymes were heterooctamers ($\alpha_4\beta_4$).

Discussion

Strain 3-2W4 was recently isolated because of its ability to degrade the β-peptides H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH, and a novel β-peptidyl aminopeptidase (BapA) was identified as the key enzyme in the degradation pathway of these non-natural β-peptides [17]. Partial 16S rDNA sequence analysis of strain 3-2W4 showed that *S. microcystinivorans* Y2 was the closest match, with 100% identity [17,21]. Strain Y2 is able to grow on microcystin, a cyclic, toxic heptapeptide that is produced by cyanobacteria and contains β-peptide structures. Strains 3-2W4 and Y2 both belong to the family *Sphingomonadaceae* [17]. Therefore, we also checked strain Y2 for the ability to use the β-peptides H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH as growth substrates. Strain Y2 grew on these β-peptides, and degradation proceeded along the same metabolic pathway as was described for strain 3-2W4 [17]. The same metabolites were detected, but strain Y2 grew more slowly on H-βhVal-βhAla-βhLeu-OH and much faster on H-βhAla-βhLeu-OH than did strain 3-2W4. The identification of a gene sequence with high similarity to *bapA* from strain 3-2W4 was, in conjunction with the growth experiments, a strong indication that the degradation of the β-peptides was also initiated by a β-peptidyl aminopeptidase in strain Y2. The heterologous expression, purification and characterization of this enzyme provided clear evidence that, indeed, a β-peptidyl aminopeptidase (Y2 BapA) was responsible for degradation of the tested β-peptides. Interestingly, the growth rates of strain 3-2W4 and strain Y2 on H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH correlated well with the specific activities of the purified 3-2W4 BapA and Y2 BapA for these two substrates (Table 2). This observation indicates that, in both cases, metabolism of the tested β-peptides was exclusively initiated by these enzymes. Although the two strains 3-2W4 and Y2 have nearly identical 16S rDNA sequences, they do not belong to the same species, as proven by DNA·DNA hybridization experiments [18]. Nevertheless, the two *bapA* genes were clustered with similar flanking genes coding for putative sugar transporters and threonine hydratases.

Analysis of the sequences of 3-2W4 BapA and Y2 BapA showed that they belong to the S58 serine peptidase family [30]. Together with the L-aminopeptidase-D-amidase/D-esterase from *O. anthropi* LMG7991 (DmpA) [22] and the β-Ala-Xaa-dipeptidase from *Pseudomonas* sp. MCI3434 (Ps BapA) [23], they form a group of peptidases with very unusual substrate specificities. The presence of a putative signal peptide is a distinctive feature of 3-2W4 BapA and Y2 BapA, because neither DmpA nor Ps BapA possess a signal sequence. All four enzymes have an $(\alpha\beta)_4$ quaternary structure [23,29]. They are inhibited neither by chelating agents nor by specific protease and peptidase inhibitors such as leupeptin, bestatin and phenylmethanesulfonyl fluoride [22,23], but the activity of 3-2W4 BapA and Y2 BapA was completely inhibited in the presence of the typical serine proteases inhibitor Pefabloc SC. In contrast, DmpA was not inhibited by Pefabloc SC [22].

The crystal structure of DmpA was elucidated, and a reaction mechanism involving Tyr146 and Asn218 for stabilization of the putative tetrahedral intermediate in the oxyanion hole was proposed for this enzyme [29]. Alignment of the amino acid sequences showed that one of the two residues that form the oxyanion hole in 3-2W4 BapA (Leu164) and Y2 BapA (Leu161) is different from that in DmpA (Tyr146) and Ps BapA (Tyr129), whereas the second residue is conserved in all four proteins (Asn218 in DmpA) (Fig. 1).

The BapA enzymes from strains 3-2W4 and Y2 have unique substrate specificities that clearly distinguish them from DmpA and BapA from *Pseudomonas* sp. MCI3434 [22,23]. They did not accept α-amino acids at the *N*-terminus; instead, they exclusively cleaved a variety of β-amino acids with proteinogenic side chains from peptide substrates. For these reactions, the following preferences were observed: aliphatic (βhAla, βhVal, βhLeu) and aromatic amino acids (βhPhe, βhTyr, βhTrp) > OH-containing amino acids (βhSer, βhThr) > βhGly and basic/polar amino acids (βhHis, βhLys, βhArg, βhGln). However, βhPro and βhGlu were not released by the two enzymes (Table 2). The kinetic parameters for most of these substrates could not be determined, due to limited availability and/or low solubility of the peptides. This fact prevents a rigorous comparison of the substrate specificities in terms of k_{cat}/K_m values. However, our results show clear and distinct preferences for cleavage of certain β-homoamino acids by the two BapA enzymes.

Whereas DmpA and Ps BapA were able to hydrolyze the chromogenic substrates H-D-Ala-*p*NA and H-Ala-*p*NA with fairly high activities, 3-2W4 BapA and Y2 BapA did not accept these compounds as substrates. Peptides with an *N*-terminal βhGly such as carnosine and H-βhGly-Ile-βhTyr-OH were not cleaved very efficiently by 3-2W4 BapA and Y2 BapA, whereas Ps BapA exhibited high activities for dipeptides and amides carrying an *N*-terminal βhGly [23]. These observations were supported by the rather low catalytic efficiencies of 3-2W4 BapA and Y2 BapA for H-βhGly-*p*NA (Table 3).

Both β-peptidyl aminopeptidases (3-2W4 BapA and Y2 BapA) are key enzymes in the microbial degradation of non-natural β-peptides. The investigation of the substrate specificities showed that both enzymes catalyze similar reactions. 3-2W4 Bap exhibited higher activities towards the majority of the tested peptides than did Y2 BapA, whereas the latter hydrolyzed peptides with small *N*-terminal β-homoamino acids more quickly.

Our results clearly show that these novel β-peptidyl aminopeptidases have stringent requirements for potential peptide substrates with regard to the structure of the peptide backbone; peptides with *N*-terminal α-amino acids, which lack the additional methylene group common to β-amino acids, are not hydrolyzed at all. The requirements with regard to the structure of the variable side chains are less pronounced. Although β-peptides with *N*-terminal aliphatic and aromatic β-homoamino acids are preferred substrates, peptides with other β-homoamino acids at that position are also turned over by the enzymes. Future work will focus on structural analysis of these enzymes, on the identification and manipulation of the functionally important amino acids, and on the elucidation of the mechanism of the catalytic reaction.

Experimental procedures

Chemicals

The peptides H-βhVal-βhAla-βhLeu-OH and H-βhAla-βhLeu-OH were synthesized as previously described [16,17]. The other peptidic substrates were prepared by solid-phase peptide synthesis on a Wang-resin, starting from commercially available Fmoc-protected amino acid building blocks. Purification by preparative RP-HPLC and lyophilization yielded the corresponding tripeptides with purities above 95%. The β-peptides were designated according to the rules outlined by Seebach *et al.* [2]. It needs to be pointed out that the β-amino acid commonly known as β-Ala is named βhGly according to this nomenclature. Unless otherwise specified, all peptides and amino acid-containing substrates were solely composed of L-amino acids, and all β-homoamino acids carried the side chain at the β^3-carbon atom. H-βhGly-*p*NA was obtained from Bachem (Bubendorf, Switzerland) and Pefabloc SC was obtained from Roche (Basel, Switzerland). All other chemicals and reagents used were of analytical grade and were purchased from Sigma-Aldrich (Buchs, Switzerland) or Merck KGaA (Darmstadt, Germany).

DNA techniques and sequence analysis

Genomic DNA of strain Y2 was isolated with the Aqua-Pure genomic DNA isolation kit (Bio-Rad, Reinach, Switzerland) and applied as template in PCR experiments for the amplification of a part of the *bapA* gene. The PCR was performed with the degenerated primers for_35 (5′-TTC GARCCGACSCCSGGCGC-3′) and rev_36 (5′-GCRTC SGTSGCGATSACGAT-3′), as described previously [17]. The complete *bapA* gene sequence was isolated with the Universal GenomeWalker kit (BD Biosciences, Basel, Switzerland), according to the instructions of the manufacturer. The GenomeWalker adaptors were ligated to DNA fragments that were produced by digestion with *Pvu*II, *Eco*RV,

*Stu*I, and *Nru*I. These libraries were used in nested PCR experiments with the Expand Long Template PCR System (Roche, Mannheim, Germany) and the following primers: Na_64 (5′-CTGAAATGACCGTGGCGTGGC-3′), Ni_66 (5′-CGCAACGTCGGTGATGGCATTC-3′), Ca1-65 (5′-GGACAGGGATGCATCTCGTCG-3′), Ci1-67 (5′-ATC GTTCCTGGGGCCGGTCG-3′), Ca2-68 (5′-AATT CGCTGCTGATCGTGATCGCCACA-3′), and Ca2-69 (5′-GATGCACCGCTGATGCCGCATCAGCTG-3′). For cloning purposes, the *bapA* gene sequences from strains 3-2W4 and Y2 were amplified from genomic DNA with primers containing the restriction sites for *Nde*I and *Bam*HI (3-2W4-51, 5′-GGAATTCATATGGGGCCGCGC GCTCGCGATCT-3′; 3-2W4-42, 5′-CGGATCCTACCGG CGCGGAAACCGCGCCT-3; Y2–70, 5′-GGAATTCCAT ATGGGTCCGCGCGCACG-3′; Y2–71, 5′-CGGATCCTA TCGGCGCGGGAACCG-3′; the restriction sites are underlined). The PCR products were restricted with *Nde*I and *Bam*HI and cloned in the expression vector pET3c (EMD Bioscience Inc., San Diego, CA, USA) cut with the same enzymes creating the plasmids p3BapA and pYBapA. Then, plasmids were transformed into *E. coli* BL21(DE3) pLysS (Novagen). All DNA sequencing reactions were carried out by Sequiserve (Vaterstetten, Germany) with an ABI 3730 sequencer.

The gene copy number of *bapA* from strain 3-2W4 was determined by Southern blot. Six hundred and forty base pairs of the *bapA* gene were amplified with the primers ES_f_9 (5′-GCACTAGTCGTGCACCAGAGTATGATG G-3′) and ES_r_10 (5′-GGAATTCATATGCTGTCGGT GTCGTTGATGAT-3′), and labeled with digoxigenin by incubation at 37 °C for 20 h. Labeling and hybridization were performed with the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche, Mannheim, Germany). Digested genomic DNA was resolved on a 1% (w/v) agarose gel, transferred onto a Hybond N+ membrane (GE Healthcare Bioscience AB, Uppsala, Sweden) by vacuum blotting, and probed with the digoxigenin-labeled *bapA* fragment.

Media and growth conditions

Strain Y2 was cultivated in minimal media containing 5 mM H-βhVal-βhAla-βhLeu-OH or H-βhAla-βhLeu-OH as sole source of carbon, nitrogen and energy. The exact compositions of both media (TriMM2 and DiMM2) have been reported previously [17]. Nutrient broth and nutrient agar served as nonselective complex media (Biolife, Milan, Italy). Cultivations of strain Y2 were performed at 25 °C and 200 r.p.m. The recombinant *E. coli* BL21(DE3) pLysS strains carrying p3BapA or pYBapA were cultivated in a KLF 2000 Bioreactor (Bioengineering AG, Wald, Switzerland). The batch medium contained 13.3 g of KH_2PO_4, 4.0 g of ammonium sulfate, 1.7 g of citric acid, 20 g of glucose, 2 mM magnesium sulfate, 50 mg of ampicillin and 25 mg of chloramphenicol per liter. One liter of medium was supplemented with 5 mL of a trace element solution containing 1 mol of HCl, 1.5 g of $MnCl_2$ $4H_2O$, 1.05 g of $ZnSO_4$, 0.3 g of H_3BO_3, 0.25 g of Na_2MoO_4 $2H_2O$, 0.15 g of $CuCl_2$ $2H_2O$, 0.84 g of $Na_2EDTA.2H_2O$, 4.12 g of $CaCl_2$ $2H_2O$ and 4.87 g of $FeSO_4$ $7H_2O$ per liter. The pH of the batch medium was set to 7.4 with ammonia. The cells for the inoculum were grown in 200 mL of M9 minimal medium with 2% (w/v) glucose, centrifuged for 10 min at 10000 ***g*** and 4 °C, and suspended in 10 mL of saline. The feeding solution contained 73% (w/v) glucose and 19.6 g of $MgSO_4.7H_2O$ per liter. The batch volume was 2 L and the feed volume was 0.27 L. After 13 h of fermentation, the feed of strain *E. coli* BL21(DE3) pLysS carrying p3BapA was started, and 100 mg of ampicillin was added to the batch medium. The cells were induced with 2 mM isopropyl thio-β-D-galactoside, and the temperature was reduced from 37 °C to 30 °C after 16.5 h. After 3 more hours, the fermentation was stopped and the cells were harvested by centrifugation. The cultivation of strain *E. coli* BL21(DE3) pLysS harboring pYBapA was carried out in the same way, but following a slightly different time line. For this strain, the feed was started after 15 h of fermentation; the cells were induced after 18.3 h and harvested after 21.5 h.

Protein purification

Recombinant 3-2W4 BapA was purified by anion exchange chromatography and hydrophobic interaction chromatography according to the previously published procedure for the wild-type 3-2W4 BapA [17]. The two final steps of the protocol, the elution from the hydrophobic support and the removal of organic solvents, were modified. Recombinant 3-2W4 BapA was eluted in the presence of 30% (v/v) isopropanol in 0.5 mM Tris/HCl (pH 8.0). For the removal of isopropanol and stabilization purposes, the active fractions were lyophilized and stored at 4 °C.

Two grams of *E. coli* BL21(DE3) pLysS cells harboring pYBapA were suspended in 8 mL of 10 mM Tris/HCl (pH 8.0) (buffer A) and disrupted by ultrasonication under constant cooling on ice. After removal of the cell debris by centrifugation, 10 mL of buffer A was added to the supernatant to obtain a final dilution of 10% (w/v). All protein purification steps were performed at 4 °C. The cell extract was loaded onto a Bio-Scale column packed with Macro-Prep High Q Support (1.5 × 11.3 cm; Bio-Rad) that had previously been equilibrated with buffer A. Y2 BapA was eluted from the column with a linear gradient of buffer A and 1 M sodium chloride in 50 mM Tris/HCl (pH 8.0) (buffer B). Active fractions were pooled, and sodium chloride was added to a final concentration of 1 M. The pool was applied onto a Bio-Scale column (1.5 × 11.3 cm; Bio-Rad) packed with Phenyl Sepharose FF low sub (Amersham Biosciences AB, Uppsala, Sweden) and equilibrated with buffer B. The column was washed with buffer B and

10 mM Tris/HCl (pH 8.0). Y2 BapA was eluted with 30% (v/v) isopropanol in 0.5 mM Tris/HCl (pH 8.0). Fractions containing the active enzyme were pooled, lyophilized and stored at 4 °C. 3-2W4 BapA and Y2 BapA were analyzed by SDS/PAGE using 10% Tricine gels (Invitrogen, DH Breda, The Netherlands). Protein staining was performed with Coomassie Brilliant Blue G-250 and accelerated by heating in a microwave oven [31]. The purity of the samples was estimated using the program GENE IMAGIR 4.03.

HPLC and MS analysis

Peptides and tyrosine were quantitatively analyzed by RP-HPLC on a Dionex HPLC system equipped with a P680 pump, an ASI-100 automated sample injector, and a UVD340U photodiode array detector (Dionex, Sunnyvale, CA, USA). Samples were injected onto a Nucleosil 100-5 C_{18} column (250 × 4 mm; Macherey-Nagel, Düren, Germany). The column was equilibrated with 0.1% trifluoroacetic acid, and a gradient from 0% to 30% acetonitrile was applied within 9.8 min for the separation of the samples at a flow rate of 1 mL·min^{-1}. Peptides were detected at 205 nm and tyrosine at 275 nm. We quantified βhVal using the same HPLC system equipped with a Sumichiral OA-5000 column (150 × 4 mm; Sumika Chemical Analysis Service, Osaka, Japan) according to the method of Komeda & Asano [23]. Mass spectra of peptides were determined with an API 4000 liquid chromatography–tandem MS system connected to an Agilent 1100 LC system (Applied Biosystems, Rotkreuz, Switzerland). Molecular masses of the intact proteins and their noncovalent complexes were analyzed using a Bruker Reflex III MALDI-TOF mass spectrometer equipped with a nitrogen laser (Bruker Daltonics GmbH, Faellanden, Switzerland) and a quadrupole orthogonal time-of-flight (Q-TOF) mass spectrometer (Waters Corporation, Elstree, UK) fitted with the standard electrospray source. For MALDI-TOF MS, 0.5 μL of the protein solution was directly spotted on the target plate and mixed with 0.5 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid in methanol/acetonitrile/acetone/trifluoroacetic acid (50 : 25 : 25 : 0.1, v/v/v/v). The sample was allowed to dry, and the spectra were collected in the linear mode. Three hundred laser shots were summed per sample spectrum. For ESI MS, the buffer of the samples was exchanged for 20 μM ammonium acetate (pH 7.0), loaded into the syringe, and electrosprayed into the Q-TOF mass spectrometer. Spectra were collected until the signal reached the desired intensity. The data were processed using MASSLYNX software (Waters Corporation).

Enzyme assay

Enzyme activity was assayed by following the hydrolysis of H-βhGly-*p*NA. Unless otherwise stated, the formation of *p*NA was measured spectrophotometrically at 405 nm and 25 °C (ε = 8800 M^{-1}·cm^{-1}). The standard reaction mixture contained 5 mM H-βhGly-*p*NA, 50 mM Tris/HCl (pH 8.0), 10% (v/v) dimethylsulfoxide, and enzyme in limiting amounts. One unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmol of *p*-quiline per minute.

Kinetic measurements

The reaction rates of 3-2W4 BapA and Y2 BapA for different concentrations of the substrate H-βhGly-*p*NA (concentration range 0.5–25 mM) were measured spectrophotometrically at 25 °C. At different concentrations of the substrates H-βhVal-βhAla-βhLeu-OH, H-βhAla-βhLeu-OH and carnosine, the reaction rates were measured at 37 °C by quantification of the released amino acids βhVal, βhLeu and His, respectively, with HPLC. The kinetic parameters V_{max} and K_m were calculated with the software IGOR PRO (WaveMetrics Inc., Lake Oswego, OR, USA). The k_{cat} value was determined on the basis of the theoretical molecular mass, assuming 100% activity of the enzyme preparation. The calculation was based on weighted nonlinear regression analysis of the Michaelis–Menten model. Kinetic parameters of the other β-peptides and mixed β/α-peptides could not be measured, due to limited availability and low solubility.

Analysis of substrate specificity

The substrates were incubated with purified 3-2W4 BapA or Y2 BapA. The reaction mixtures contained 2–3 mM of the peptide, 50 mM Tris/HCl (pH 8.0), 10% (v/v) dimethylsulfoxide, and enzyme in limiting amounts. The assays were performed at 37 °C. Samples were withdrawn regularly, and the enzymatic reaction was stopped by the addition of 25% (v/v) 1 M HCl. After centrifugation, the supernatants were analyzed by HPLC.

Analysis of inhibitors

Several protease inhibitors were tested for their effects on the hydrolytic activity of the two enzymes. After preincubation of the enzymes for 30 min in 50 mM Tris/HCl (pH 8.0) and in the presence of inhibitor, the substrate H-βhVal-βhAla-βhLeu-OH was added to a final concentration of 5 mM. The preincubation step was omitted for Pefabloc SC, because this substance is not stable under slightly basic conditions. The remaining hydrolytic activity of the enzymes was determined by HPLC analysis of the residual substrate over a period of 30 min.

Size exclusion chromatography

The purified enzymes 3-2W4 BapA and Y2 BapA were applied onto a Superdex 200 column (1.6 × 47 cm;

Amersham Biosciences AB, Uppsala, Sweden) that had been equilibrated with 50 mM Tris/HCl containing 150 mM KCl (pH 8.0). Thyroglobulin (670 kDa), ferritin (440 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa) were used as standards. Recombinant DmpA from *O. anthropi* (161 kDa) was employed as an additional reference protein.

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