DOI: 10.1002/cbic.200900757

β-Aminopeptidase-Catalyzed Biotransformations of β²-Dipeptides: Kinetic Resolution and Enzymatic Coupling

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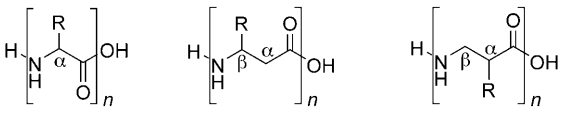
We have previously shown that the β-aminopeptidases BapA from *Sphingosinicella xenopeptidilytica* and DmpA from *Ochrobactrum anthropi* can catalyze reactions with non-natural β³-peptides and β³-amino acid amides. Here we report that these exceptional enzymes are also able to utilize synthetic dipeptides with N-terminal β²-amino acid residues as substrates under aqueous conditions. The suitability of a β²-peptide as a substrate for BapA or DmpA was strongly dependent on the size of the C_α substituent of the N-terminal β²-amino acid. BapA was shown to convert a diastereomeric mixture of the β²-peptide H-β²hPhe-β²hAla-OH, but did not act on diastereomerically pure β²,β³-dipeptides containing an N-terminal β²-homoalanine. In contrast, DmpA was only active with the latter dipeptides as substrates. BapA-catalyzed transformation of the diastereomeric mixture of H-β²hPhe-β²hAla-OH proceeded along two highly *S*-enantioselective reaction routes, one leading to substrate hydrolysis and the other to the synthesis of coupling products. The synthetic route predominated even at neutral pH. A rise in pH of three log units shifted the synthesis-to-hydrolysis ratio (v_S/v_H) further towards peptide formation. Because the equilibrium of the reaction lies on the side of hydrolysis, prolonged incubation resulted in the cleavage of all peptides that carried an N-terminal β-amino acid of *S* configuration. After completion of the enzymatic reaction, only the *S* enantiomer of β²-homophenylalanine was detected ($ee > 99\%$ for H-(*S*)-β²-hPhe-OH, $E > 500$); this confirmed the high enantioselectivity of the reaction. Our findings suggest interesting new applications of the enzymes BapA and DmpA for the production of enantiopure β²-amino acids and the enantioselective coupling of N-terminal β²-amino acids to peptides.

Introduction

β-Amino acids with proteinogenic side chains are backbone-elongated homologues of the naturally occurring proteinogenic α-amino acids (Scheme 1).[1] In contrast to their α-peptidic counterparts, peptides comprising β-amino acids are characterized by high resistance to enzymatic degradation and metabolic breakdown.[2] Properly designed β-peptides composed of β³-amino acid residues with proteinogenic side chains fold into stable secondary structures, such as the 3_{14}-helix, which was found to be the predominant secondary structure of β³-peptides.[1,3] β²-Peptides, with side chains attached to the α- instead of the β-carbon (Scheme 1), are conformationally more flexible than their β³-peptide isomers. Synthetic peptides with alternating β²- and β³-amino acid residues as well as mixed β,α-peptides revealed novel hairpin-turn-like structures that resemble the β-turns of conventional α-peptides.[4] These properties give rise to interesting new biomedical applications for β-peptides as proteolytically stable mimics of bioactive natural peptides.[5] Some recent examples of β-peptide-based designs of peptidomimetics include inhibitors of protein–protein interactions and viral cell entry,[6] ligands of the somatostatin receptor and the major histocompatibility complex (MHC),[7] as well as β-peptides with antifungal and antimicrobial activities.[8] Furthermore, a very recent study has suggested mixed α,β-peptides to be a new class of water-soluble nanoporous materials that adsorb nitrogen gas.[9]



Scheme 1. Structural comparison of α- and β-amino acid residues in peptides.

[1] *The β-amino acids used in this study are named according to the nomenclature for β-amino acids with proteinogenic side chains (βhXaa).*[1]

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900757.

β^2-Amino acids are valuable compounds for extending the repertoire of building blocks for peptide design because their incorporation into peptides induces unique secondary-structure elements and leads to increased stability against proteolytic breakdown.[10] Unlike β^3-amino acids, most of which are by now commercially available, enantiopure β^2-amino acids with proteinogenic side chains are less easily accessible by chemical synthesis. They cannot be prepared by stereoselective homologation of the naturally occurring α-amino acid analogues, the strategy most commonly applied to the synthesis of enantiopure β^3-amino acids (Arndt–Eistert reaction).[1,3,10,11] Instead, the synthesis of enantiomerically pure β^2-amino acids frequently involves multistep transformations with the use of chiral auxiliaries. Classical chemical-resolution strategies to obtain enantiopure β^2-amino acids from racemic starting materials have so far been unsuccessful.[12]

The β-aminopeptidases BapA from *Sphingosinicella xenopeptidilytica* and DmpA from *Ochrobactrum anthropi* recently aroused interest because they can catalyze the degradation of β^3-peptides with proteinogenic side chains that are otherwise stable to proteolytic breakdown.[2,13] Despite sharing an amino acid sequence identity of 42%, BapA and DmpA differ with respect to their substrate specificities. DmpA cleaves both α- and β^3-amino acids from the N termini of peptides, but shows a distinct preference for N-terminal amino acids that are unsubstituted or carry only small side chains (e.g., $-CH_3$). In contrast, BapA only catalyzes the removal of N-terminal β^3-amino acids and does not cleave peptides with N-terminal α-amino acids. However, BapA has a broader substrate specificity than DmpA as it accepts a wide variety of peptides that carry N-terminal β^3-amino acids with different proteinogenic side chains.[14] These findings demonstrated for the first time that the supposed proteolytic stability of β-peptides is not absolute. Further studies with BapA and DmpA revealed their usefulness as biocatalysts for the efficient production of enantiopure aliphatic β^3-amino acids by kinetic resolution of racemic β^3-amino acid amides,[15] and for the enzyme-catalyzed synthesis of various β^3-amino acid-containing peptides from C-terminally activated β-amino acid residues under kinetic control.[16]

The promising results obtained from the β-aminopeptidase-catalyzed conversions of β^3-peptides encouraged us to conduct enzymatic studies with β^2-amino acid-containing peptides, which have been less explored due to their limited availability. In fact, only very few enzyme-catalyzed conversions of β^2-amino acid-containing compounds have been reported, most of which describe the enantioselective production of β^2-amino acids from racemic starting materials.[17] To the best of our knowledge, the proteolytic breakdown of β^2-peptides has not yet been investigated. In this investigation, we address the potential of the β-aminopeptidases BapA and DmpA to degrade β^2-peptidic substrates as well as the stereoselectivity of these reactions. Furthermore, we describe the β-aminopeptidase-catalyzed formation of β^2-peptides under kinetic control.

Results and Discussion

Enzymatic conversion of a diastereomeric mixture of the β^2-dipeptide H-β^2hPhe-β^2hAla-OH (1)

Dissolved BapA and DmpA were tested for their ability to act on peptides composed of non-natural β^2-amino acids under aqueous conditions. We synthesized a diastereomeric mixture of the β^2-dipeptide H-β^2hPhe-β^2hAla-OH ((*S*,*S*)-**1**, (*S*,*R*)-**1**, (*R*,*S*)-**1**, and (*R*,*R*)-**1**), which allowed us to investigate the β-aminopeptidase-catalyzed breakdown of the compound and the stereoselectivity of the reaction. We separated the diastereomers of **1** on a reversed stationary phase, and quantified substrates and products of the enzymatic conversions by HPLC-UV. As the mixture of **1** contains two diastereomeric pairs of enantiomers ((*S*,*S*)-**1**, (*R*,*R*)-**1** and (*S*,*R*)-**1**, (*R*,*S*)-**1**), two reversed-phase HPLC peaks were obtained. According to the elution of a pure standard of (*S*,*S*)-**1** ($t_R = 17.1$ min) and a standard of (*S*,*S*)-**1** that contained traces of (*S*,*R*)-**1** ($t_R = 17.7$ min), peak 1 ($t_R = 17.1$ min) could be attributed to (*S*,*S*)-**1**/(*R*,*R*)-**1** and peak 2 ($t_R = 17.7$ min) to (*S*,*R*)-**1**/(*R*,*S*)-**1**.

In our investigation, we detected substrate transformation only with BapA, whereas DmpA left the mixture of **1** untouched. During the assay period at pH 7.2 and 37 °C, the decrease of **1** leveled off at approximately 57% of the initial substrate concentration of 5 mM (Figure 1). It is possible that the enzymatic reaction slowed down at the end of the 11-day incubation due to partial inactivation of the enzyme. However, the fact that both peaks of **1** decreased to approximately half of their initial areas indicated a high stereoselectivity of the BapA-catalyzed reaction. The initial decrease of the area under peak 2 ((*S*,*R*)-**1**/(*R*,*S*)-**1**) was about 1.3 times faster than the decrease of the area under peak 1 ((*S*,*S*)-**1**/(*R*,*R*)-**1**). Appropriate control experiments showed that the substrate **1** was chemically stable under the reaction conditions over the assay period.

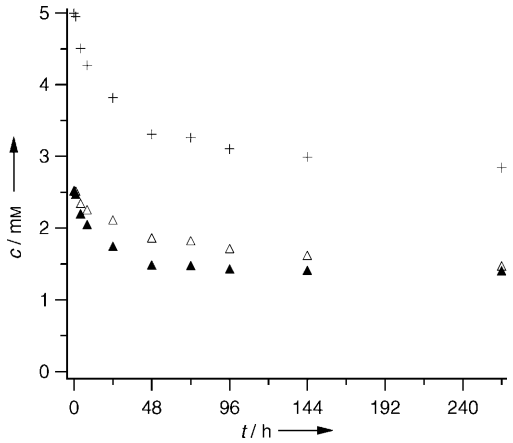


Figure 1. Conversion of H-β^2hPhe-β^2hAla-OH (**1**, +) by BapA (0.3 mg of protein per mL) at pH 7.2 and 37 °C. Two peaks of the diastereomeric mixture that correspond to (*S*,*S*)-**1**, (*R*,*R*)-**1** ($t_R = 17.1$ min, △) and to (*S*,*R*)-**1**, (*R*,*S*)-**1** ($t_R = 17.7$ min, ▲) were detected by reversed-phase HPLC. The original HPLC traces are shown in the Supporting Information (Figure S1).

Previous studies with β^3-peptides and β^3-amino acid amides showed that BapA and DmpA strongly discriminated between N-terminal β^3-amino acids that carry side chains of different sizes.[14,16a] While BapA accepts a broad range of N-terminal β^3-amino acids with different side-chain lengths and functionalities, DmpA only catalyzes reactions with small, sterically undemanding β^3-amino acids, such as N-terminal β^3-homoalanine and β-homoglycine.[2] According to our present results, this observation applies to the degradation of the β^2-dipeptide **1** as well. Structural information on the active-site compositions of BapA (unpublished results) and DmpA (PDB ID: 1B65)[18] reveal that their different substrate specificities are possibly caused by different topologies of the enzyme active sites. Whereas the active-site pocket of BapA is relatively wide, the DmpA substrate binding site is constricted by a loop region, ranging from Gln131 to Trp137, that is likely to hinder the accessibility of the active site for substrates with bulky N-terminal amino acids.

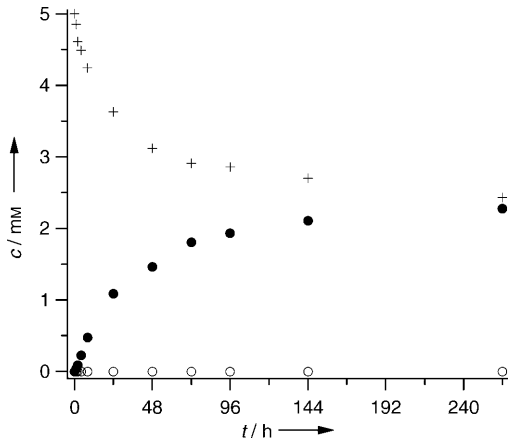


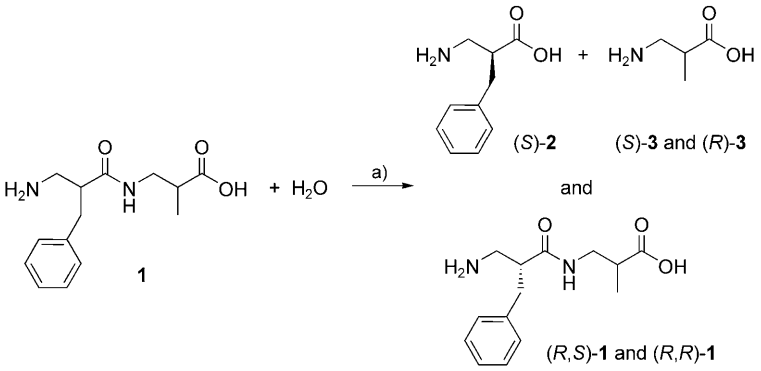
Figure 2. Formation of H-(*S*)-β^2hPhe-OH ((*S*)-**2**, ●) and H-(*R*)-β^2hPhe-OH ((*R*)-**2**, ○) from the diastereomeric mixture of 5 mM H-β^2hPhe-β^2hAla-OH (**1**, +) catalyzed by BapA (0.3 mg of protein per mL) at pH 7.2 and 37 °C. The original HPLC traces are shown in Figure S2.

Kinetic resolution of the diastereomeric pairs of enantiomers of the β^2-dipeptide 1 by BapA

The BapA-catalyzed release of H-β^2hPhe-OH (**2**) from a 5 mM solution of the β^2-dipeptide **1** at pH 7.2 was analyzed by HPLC on a chiral stationary phase. For the separation of the enantiomers of **2** we used the teicoplanin stationary phase Chirobiotic T2, which provides good separation of many chiral β^3- and β^2-amino acids.[19] We achieved baseline separation of the enantiomers of **2**, which was a prerequisite for examining the stereoselectivity of the enzyme-catalyzed reaction. Under the applied conditions, (*R*)-**2** (t_R = 11.3 min) eluted prior to (*S*)-**2** (t_R = 15.4 min). The enantiomers of the second hydrolysis product H-β^2hAla-OH (**3**) could not be separated. The four stereoisomers of **1** were partially separated under the applied conditions. According to standards of (*S*,*S*)-**1** and (*S*,*R*)-**1**, peak 2 (t_R = 31.6 min) could be attributed to (*S*,*R*)-**1** and peak 4 (t_R = 36.0 min) to (*S*,*S*)-**1**.

Over the reaction period of 11 days, (*S*)-**2** accumulated to a concentration of 2.3 mM, which corresponds to a conversion of 46% of the initially employed β^2-dipeptide **1** (Figure 2). The reaction was highly *S*-enantioselective ($ee > 99\%$ and $E > 500$),[20] and the release of (*R*)-**2** over the assay period remained below the detection limit of 0.01 mM.

These results indicate almost quantitative conversion of both diastereomers with an N-terminal (*S*)-β^2-homophenylalanine residue ((*S*,*S*)-**1** and (*S*,*R*)-**1**) (Scheme 2). Analysis of the same samples by reversed-phase HPLC showed that (*S*,*R*)-**1** was converted 1.3-times faster than (*S*,*S*)-**1** (Figure 1). Hence, we conclude that the configuration of the N-terminal β^2-homophenylalanine residue is crucial for the successful conversion of **1** by BapA, whereas the configuration of the second amino acid in the β^2-dipeptide is only of minor importance.

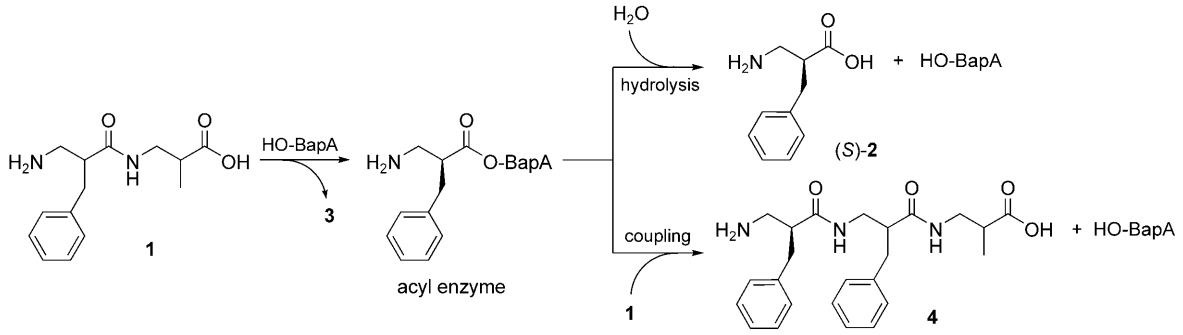


Scheme 2. Kinetic resolution of the diastereomeric pairs of enantiomers of the β^2-dipeptide **1** by BapA. a) BapA, 11 days, pH 7.2, 37 °C.

BapA-catalyzed peptide coupling reactions with the β^2dipeptide 1

The BapA-catalyzed conversion of the β^2-dipeptide **1** was characterized by simultaneously occurring hydrolysis and coupling reactions among substrates and products (Scheme 3). This complex interplay of competing reactions is based on the postulated general catalytic mechanism of β-aminopeptidases,[21] which was previously described for other enzyme members of the N-terminal nucleophile (Ntn) hydrolase family, including DmpA.[18,22] Accordingly, the first step in substrate conversion is the formation of a characteristic acyl enzyme complex through nucleophilic attack of the enzyme's catalytically active serine residue on the carbonyl carbon atom of the substrate's amide bond. The acyl moiety is subsequently released from the acyl enzyme by the action of a nucleophile. The nucleophile can be either water, which leads to hydrolysis of the acyl enzyme, or the deprotonated N terminus of an amino acid or a peptide, which results in peptide bond formation. The creation of a potent nucleophile requires alkaline conditions to keep the attacking amino group in its deprotonated state.

[2] *β-Homoglycine is commonly referred to as β-alanine.*



Scheme 3. BapA-catalyzed routes for the conversion of the β²-dipeptide H-β²hPhe-β²hAla-OH (**1**) at the starting point of the reaction. All the products formed by the initial hydrolysis and coupling reactions can further react as nucleophiles. This leads to the formation of additional peptidic coupling products (**5**–**7**, see Table 1).

For the BapA-catalyzed coupling reactions with dipeptide **1**, we used an alkaline aqueous reaction system at pH 10, which had previously been shown to support peptide couplings by β-aminopeptidases.[16] At the initial stage of the reaction we observed substrate hydrolysis and the formation of the β²-tripeptide H-[β²hPhe]$_2$-β²hAla-OH (**4**; Scheme 3). This is due to the fact that water and the substrate itself were the only nucleophiles to release the acyl moiety from the active site of BapA. As the reaction proceeded, all products created by hydrolysis and coupling reactions could further react as nucleophiles themselves, which gave rise to the additional coupling products **5**–**7** (Table 1 and Figure 3).

Table 1. HPLC-MS analysis of the hydrolysis and coupling products that accumulated after 48 hours of the BapA-catalyzed conversion of the diastereomeric mixture of **1** at pH 10 and 37 °C (see also Figure 3).

Compound	t_R [min]	Calcd mass	m/z[a] $[M+H]^+$	$[M+Na]^+$
H-β²hPhe-OH (**2**)	16.6	179.1	180.2	n.d.[b]
H-β²hPhe-β²hAla-OH (**1**)	17.1	264.2	265.1	287.2
	17.7		265.1	287.3
H-[β²hPhe]$_2$-β²hAla-OH (**4**)	22.4	425.2	426.2	448.2
	22.8		426.1	448.3
	23.1		426.2	448.3
H-[β²hPhe]$_2$-OH (**5**)	23.2	340.2	341.4	363.2
H-[β²hPhe]$_3$-β²hAla-OH (**6**)	25.7	586.3	587.5	609.8
	25.9		587.5	609.5
	26.1		587.3	609.7
	26.4		587.4	609.8
H-[β²hPhe]$_3$-OH (**7**)	26.8	501.3	502.6	524.2

[a] The detected mass/charge ratios (m/z) of the hydrogen and sodium adducts of the molecular ions are given. Further fragmentations of the peptides are not shown. [b] Not detected.

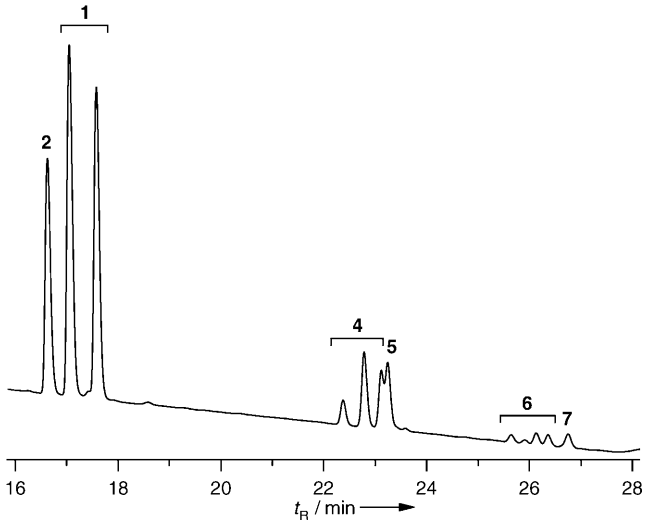


Figure 3. HPLC-UV trace of a sample taken after 48 hours from the BapA-catalyzed conversion of the diastereomeric mixture of H-β²hPhe-β²hAla-OH (**1**) at pH 10 and 37 °C. The chromatogram shows the hydrolysis product H-(*S*)-β²hPhe-OH ((*S*)-**2**) as well as the coupling products **4**–**7** (see also Table 1).

Since the overall equilibrium of the BapA-catalyzed reaction lies on the side of the hydrolysis products, all the observed coupling products that temporarily accumulated in the reaction mixture were finally hydrolyzed to their amino acid constituents β²-homophenylalanine (**2**) and β²-homoalanine (**3**; Figure 4). In contrast to **2**, the second hydrolysis product **3** could not be analyzed by reversed-phase HPLC, as the compound coeluted from the column with the flow through. Similarly to the reaction at pH 7.2, only (*S*)-**2** was detected by HPLC on chiral column material after completion of the enzymatic reaction at pH 10, and the concentration of (*R*)-**2** remained below the detection limit. After the reaction period of 10 days, the final concentration of (*S*)-**2** (2.3 mM) corresponded to substrate conversion of almost 50%.

From these data we conclude 1) that the unconverted diastereoisomers correspond to (*R*,*S*)-**1** and (*R*,*R*)-**1** and 2) that the acyl enzyme could only be formed when the N-terminal β²-homophenylalanine residue of the peptide had the *S*-configuration (Scheme 3). Consequently, all coupling products **4**–**7** formed through enzymatic conversion must share the presence of one or more N-terminal β²-homophenylalanine residues of *S* configuration. For the first coupling product H-(*S*)-β²hPhe-β²hPhe-β²hAla-OH (**4**), only three peaks were detected for the four possible diastereoisomers ((*S*,*S*,*S*)-**4**, (*S*,*S*,*R*)-**4**, (*S*,*R*,*S*)-**4** and (*S*,*R*,*R*)-**4**) by reversed-phase HPLC (Figure 3 and

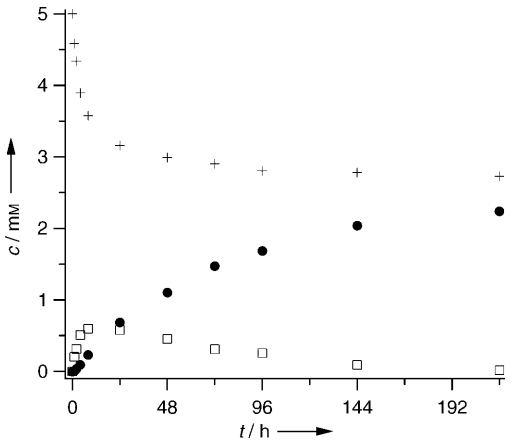
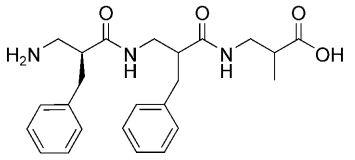
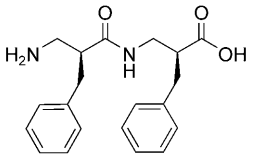


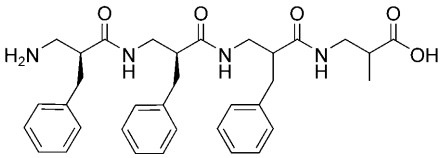
Figure 4. Conversion of the β^2-dipeptide H-β^2hPhe-β^2hAla-OH (**1**, +) at a concentration of 5 mM by BapA (0.3 mg of protein per mL) at pH 10 and 37 °C. Competing hydrolysis and coupling reactions gave rise to the formation of H-(*S*)-β^2hPhe-OH ((*S*)-**2**, ●) and various peptidic coupling products (**4**–**7**, □), respectively. Due to a lack of standards for the quantification of compounds **4**–**7**, the overall concentration of coupling products was estimated on the basis of the experimentally determined concentrations of **1** and **2**, assuming that one equivalent of coupling product is formed from two equivalents of **1**.



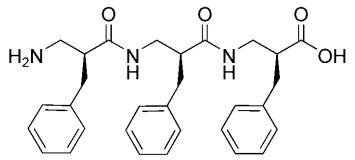
(*S*,*S*,*S*)-**4**, (*S*,*S*,*R*)-**4**, (*S*,*R*,*S*)-**4** and (*S*,*R*,*R*)-**4**



(*S*,*S*)-**5**



(*S*,*S*,*S*,*S*)-**6**, (*S*,*S*,*S*,*R*)-**6**, (*S*,*S*,*R*,*S*)-**6** and (*S*,*S*,*R*,*R*)-**6**



(*S*,*S*,*S*)-**7**

Table 1). However, the appearance of four peaks for the diastereoisomers of the tetrapeptide H-[(*S*)-β^2hPhe]$_2$-β^2hPhe-β^2hAla-OH (**6**) implies that the four possible diastereoisomers of the precursor tripeptide **4** must have been present in the reaction mixture. This is supported by the fact that the ratio of the three peak areas of **4** was approximately 1:2:1; this suggests that two of the four diastereoisomers of **4** coeluted from the column. Only one peak was detected by reversed-phase HPLC for each of the β^2-homophenylalanine oligomers **5** and **7**, which temporarily accumulated in the reaction mixture (Figure 3). As (*R*)-**2** is not present in the reaction mixture to attack the acyl enzyme, we conclude that **5** and **7** are solely composed of β^2-amino acid residues of *S* configuration.

pH-Dependence of BapA-catalyzed hydrolysis and coupling reactions with the β^2-dipeptide 1

The influence of pH on the BapA-catalyzed conversion of the diastereomeric mixture of **1** (5 mM) was investigated at pH 7.2, 8 and 10. From the initial data points obtained by reversed-phase HPLC analysis, we calculated the enzymatic rates for the overall conversion of the substrate **1** (v_C) as well as the rates for the formation of the hydrolysis product **2** (v_H) and of the coupling product **4** (v_S; Table 2). As expected from a previous investigation on the pH profile of BapA,[13c] the highest conversion rate was observed at pH 8 ($v_C = 0.021$ μmol min^{-1} per mg of protein). The v_S/v_H ratio was strongly dependent on the pH of the reaction system. Interestingly, peptide synthesis predominated over substrate hydrolysis in the initial stage of the reaction under all pH conditions. A rise in pH from 7.2 to 10 caused a further shift towards coupling product formation, as indicated by an increase in v_S/v_H from 2.4 to 10.8. Generally, the conversion of **1** catalyzed by BapA was one to two orders of magnitude slower than the conversion of β^3-amino acid-containing substrates under similar reaction conditions.[14]

Table 2. Initial rates [μmol min^{-1} per mg protein] of the BapA-catalyzed hydrolysis and coupling reactions with substrate **1** (5 mM) at 37 °C and at different pH values.[a]

pH	v_C	v_H	v_S	v_S/v_H	c_{max} [mM]
7.2	0.013	0.0022	0.0053	2.4	0.28
8	0.021	0.0026	0.0094	3.6	0.46
10	0.017	0.00075	0.0081	10.8	0.60

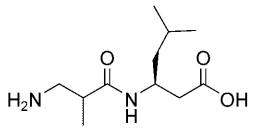
[a] The conversion rate (v_C) relates to the initial decrease in substrate, whereas the hydrolysis (v_H) and synthesis (v_S) rates relate to the initial increase of free **2** and coupling product **4**, respectively. c_{max}, which represents the maximum accumulation of all coupling products **4**–**7** in the reaction mixture, was estimated by assuming that 1 equiv of the coupling product is formed by the condensation of 2 equiv of **1**.

While the accumulation of the coupling products **4**–**7** was very low at pH 7.2 (0.28 mM), we observed maximum coupling product concentrations of 0.46 mM at pH 8 and 0.60 mM at pH 10. These concentrations correspond to conversions of 18.4 and 24%, respectively, of the initial dipeptide **1** (Table 2). Coupling product accumulation peaked in a clear-cut maximum, after which peptide hydrolysis became the predominant reaction (see Figure 4). After completion of the reactions, the only remaining detectable compounds were (*S*)-**2** and the uncon-

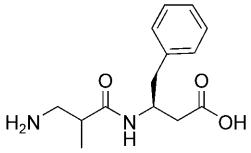
verted stereoisomers of the substrate, which correspond to (*R*,*S*)-**1** ($t_R = 28.8$ min) and (*R*,*R*)-**1** ($t_R = 33.5$ min) as determined by HPLC on the chiral column material.

Enzymatic degradation of β^2,β^3-dipeptides with N-terminal β^2-homoalanine (8 and 9)

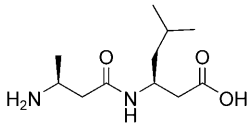
In addition to the diastereomeric mixture of the β^2-dipeptide **1**, we performed experiments with the β-aminopeptidases BapA and DmpA and four diastereomerically pure β^2,β^3-dipeptides carrying an N-terminal β^2-homoalanine and a C-terminal (*S*)-β^3-homoamino acid residue ((*S*,*S*)-**8**, (*R*,*S*)-**8** and (*S*,*S*)-**9**, (*R*,*S*)-**9**).



(*S*,*S*)-**8** and (*R*,*S*)-**8**



(*S*,*S*)-**9** and (*R*,*S*)-**9**



(*S*,*S*)-**10**

Interestingly, we did not detect any conversion of substrates **8** and **9** with BapA, whereas DmpA cleaved all four diastereoisomers. This is in contrast to our observations with substrate **1** and to previous results with H-(*S*)-β^3hAla-(*S*)-β^3hLeu-OH ((*S*,*S*)-**10**), which is the β^3-dipeptide isomer of (*S*,*S*)-**8**.[13c,14] The results shown in Table 3 indicate that the catalytic rates for the DmpA-catalyzed conversion of (*S*,*S*)-**8** and (*R*,*S*)-**8** were 70 and 90 times lower than the conversion rate for the corresponding β^3-dipeptide (*S*,*S*)-**10** determined in this study under the same conditions. An exchange of the C-terminal β^3-homoleucine residue of **8** by the more hydrophobic β^3-homophenylalanine of **9** had an accelerating effect on substrate conversion by DmpA. DmpA converted dipeptides **8** and **9** with N-terminal β^2-homoalanine residues of both *S* and *R* configuration with low stereoselectivity, which is in contrast to the previously reported highly *S*-enantioselective kinetic resolution of racemic β^3-homoalanine amide and β^3-homoalanine *p*-nitroanilide by the same enzyme.[15]

Table 3. Conversion rates [μmol min^{-1} per mg of protein] of the diastereomerically pure substrates **8**, **9** and **10** (5 mM) by DmpA at pH 7.2 and 37 °C.

Substrate	Conversion rate	Substrate	Conversion rate
(*S*,*S*)-**8**	0.029	(*R*,*S*)-**8**	0.037
(*S*,*S*)-**9**	0.099	(*R*,*S*)-**9**	0.48
(*S*,*S*)-**10**	2.5		

Conclusions

In this investigation we have studied transformations of non-natural peptides with N-terminal β^2-amino acid residues under aqueous reaction conditions by using the β-aminopeptidases BapA and DmpA as catalysts. Our study describes for the first time how peptides composed of β^2-amino acids with proteinogenic side chains can be converted by hydrolytic enzymes, though with low catalytic rates when compared to β^3-peptidic substrates.[14] As previously observed for the conversion of β^3-peptides, the enzymes had distinct substrate preferences depending on the size of the side chain on the N-terminal amino acid. Only BapA acted on substrate **1** with its bulky N-terminal β^2-homophenylalanine residue, whereas the conversion of peptides **8** and **9**, which carry N-terminal β^2-homoalanine and C-terminal β^3-homoleucine or β^3-homophenylalanine residues, was only catalyzed by DmpA. While DmpA converted substrates with N-terminal amino acids of *S* and *R* configuration, the BapA-catalyzed reaction was highly *S* enantioselective. BapA simultaneously catalyzed hydrolysis and synthesis reactions with the diastereomeric mixture of **1**, leading to the hydrolysis of the substrate and to the formation of various coupling products. The ratio of the rate of peptide formation to the rate of peptide hydrolysis was positively correlated with an increase in the pH.

The availability of enantiopure β^2-amino acids is limited because chemical preparations of these compounds involve labor- and cost-intensive multistep reactions.[12] Hence, alternative enzymatic approaches to the enantioselective production of β^2-amino acids are highly desirable to complement chemical asymmetric-synthesis strategies. The highly *S* enantioselective reactions of BapA with a model β^2-dipeptide indicate that β-aminopeptidases could become useful for the biocatalytic production of enantiopure β^2-amino acids by the kinetic resolution of racemic β^2-amino acid derivatives, for example, β^2-amino acid amides or esters. Furthermore, the enzymes could be used to catalyze the introduction of a β^2-amino acid residue as an N-terminal protecting group into a peptide, thereby stabilizing the peptide against degradation by other exopeptidases. To fully assess the biocatalytic potential of BapA and DmpA for enantioselective conversion of β^2-peptidic substrates, it will be necessary to 1) optimize the present reaction system with the aim of suppressing uncontrolled cross-couplings among substrates and products and 2) carry out a detailed analysis of the enzymes' substrate specificities for peptides and amides carrying N-terminal β^2-amino acids with different side chains.

Experimental Section

General remarks: The amino acids and peptides **1**–**10** were analyzed on a Dionex HPLC system equipped with a P680 pump, an ASI-100 automated sample injector, an UltiMate 3000 thermostatted column compartment, and a UVD 340U photodiode array detector (Dionex, Sunnyvale, CA, USA). Enantiomers of the β^2-amino acid H-β^2hPhe-OH (**2**) were separated without further derivatization on the chiral teicoplanin stationary phase Chirobiotic T2 (250× 4.6 mm; Astec, Whippany, NJ, USA) at a constant temperature of

10 °C and quantified by relating the absorbance at 205 nm to a standard curve of enantiopure (*R*)-**2**. The mobile phase was methanol/H_2O (90:10), and the applied flow rate was 1 mL min^{-1}. Under the described separation and detection conditions, the detection limit for **2** was 0.01 mM.

Additionally, all samples were analyzed by reversed-phase HPLC on a Nucleodur C_{18}-Pyramid stationary phase (250×4 mm, 5 μm particle size; Macherey–Nagel, Düren, Germany), which was equilibrated with 0.1% aqueous trifluoroacetic acid (TFA) at a column temperature of 20 °C. All substrates and products were separated with a linear gradient of 0 to 40% acetonitrile at a constant flow rate of 1 mL min^{-1} and detected at a wavelength of 205 nm. Coupling products were quantified on basis of the experimentally determined concentrations of the compounds **1** and **2** on the assumption that one molecule of coupling product is formed from two molecules of substrate. Mass spectra of all compounds were recorded with an API 4000 liquid chromatography/tandem MS system connected to an Agilent 1100 LC system. For protein determination, we used fivefold concentrated Bradford reagent (Bio-Rad, Rheinach, Switzerland) and bovine serum albumin (BSA) as a standard; absorbance measurements were performed at 595 nm with a Specord S100 spectrophotometer (Analytik Jena, Jena, Germany).

Enzyme expression and purification: The genes *bapA* and *dmpA*, which code for the β-aminopeptidases BapA from *S. xenopeptidilytica* 3-2W4 and DmpA from *O. anthropi* LMG7991, respectively, were cloned into the expression plasmid pET9c (Novagen, Madison, USA). Plasmid p3BapA[13c] was digested with BamHI and NdeI. The *bapA*-containing fragment was cloned into pET9c, which was cut at the same sites to yield pAR116. The *dmpA* gene was cloned from pODmpA[14] into pET9c according to the same procedure with the restriction enzymes BamHI and SalI to yield pAR114. The two obtained expression plasmids were transformed into *E. coli* BL21(DE3) pLysS (Novagen, Madison, USA) and recombinantly expressed in the presence of kanamycin and chloramphenicol. BapA and DmpA were purified according to established procedures.[13b,14] The lyophilized enzyme powders were dissolved in a potassium phosphate buffer (10 mM, pH 7.2), and the protein contents of the enzyme stocks were determined spectrophotometrically.

General procedure for the enzyme-catalyzed conversion of the diastereomeric mixture of H-β^{2}hPhe-β^{2}hAla-OH (1): The reaction mixtures contained the diastereomeric mixture of **1** (5 mM) in solutions of different buffering systems (100 mM, potassium phosphate pH 7.2, Tris/HCl pH 8, sodium carbonate pH 10). Enzymatic conversions of the substrates were initiated by the addition of an appropriate amount of BapA or DmpA. Samples were withdrawn at intervals from the reaction mixtures, and the enzymatic reaction was quenched by heating the samples at 90 °C for 3 min. The reaction products were analyzed by chiral and reversed-phase HPLC. A sample taken from the enzymatic assay at pH 10 after 48 h was subjected to HPLC-MS analysis.

General procedure for the enzyme-catalyzed conversion of the enantiopure substrates 8, 9 and 10: The reaction mixtures contained 5 mM of the diastereomerically pure β^2,β^3-dipeptides H-(*S*)-β^2hAla-(*S*)-β^3hLeu-OH ((*S*,*S*)-**8**), H-(*R*)-β^2hAla-(*S*)-β^3hLeu-OH ((*R*,*S*)-**8**), H-(*S*)-β^2hAla-(*S*)-β^3hPhe-OH ((*S*,*S*)-**9**), H-(*R*)-β^2hAla-(*S*)-β^3hPhe-OH ((*R*,*S*)-**9**), or the β^3-dipeptide H-(*S*)-β^3hAla-(*S*)-β^3hLeu-OH ((*S*,*S*)-**10**)[13c] in potassium phosphate buffer (100 mM, pH 7.2) containing 10% DMSO. Enzymatic conversions of the substrates were carried out as described above, and samples were analyzed by reversed-phase HPLC.

Acknowledgements

This work was supported by the Swiss National Science Foundation (Project No. 3152A0-100770, B.G.), the Deutsche Bundesstiftung Umwelt (Project No. 13176-32, T.H.) and the New Zealand Foundation for Research Science and Technology (No. SWSS0401, J.G.).

Keywords: aminopeptidases · hydrolases · kinetic resolution · nucleophiles · peptides

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Received: December 11, 2009
Published online on March 25, 2010

