The Outstanding Biological Stability of β- and γ-Peptides toward Proteolytic Enzymes: An In Vitro Investigation with Fifteen Peptidases

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*A series of 36 linear and cyclic β- and γ-peptides consisting of as few as two, and as many as 15 residues, was offered as substrates to 15 commercially available proteases of bacterial, fungal, and eukaryotic origin, including a β-lactamase and amidases, as well as most vigorous, nonspecific proteases, such as the 20S proteasome from human erythrocytes. For comparison, an α-eicosapeptide and standard substrates of the proteolytic enzymes were included in the investigation. Under conditions of complete cleavage of the α-peptide within 15 min the β- and γ-peptides were stable for at least 48 h. Inhibition studies with seven β- and γ-peptides and α-chymotrypsin show that the residual enzyme activity toward succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide is unchanged within experimental error after incubation for 15 min with the peptide analogues. Thus, β- and γ-peptides with proteinogenic side chains, that is, consisting of the singly or doubly homologated natural α-amino acids (one or two CH_2 groups inserted in the backbone of each residue), are completely stable to common proteases, without inhibiting their normal activity (as demonstrated for α-chymotrypsin). This proteolytic stability of peptides built of homologated amino acids is a prerequisite for their potential use as drugs.*

KEYWORDS:

hydrolases · peptidases · peptides · peptidomimetics · proteolysis

Introduction

Despite the central function of peptides and proteins in all living systems, the use of peptides as drugs and immunogens is severely hampered by their low bioavailability and by their susceptibility to enzymatic degradation. Moreover, the inherent flexibility of the peptide backbone usually makes the bioactive conformation of a peptide poorly defined. Thus, it is not surprising that considerable efforts have been made, both in academia and in the pharmaceutical industry, to overcome these difficulties.[1, 2]

A number of strategies have been suggested to increase enzymatic stability of peptides, one possibility being the incorporation of β-amino acids: As suggested already at the beginning of the last century by Abderhalden et al., peptide bonds involving β-amino acids should be resistant to enzymatic cleavage.[3, 4] Although this first report was subject to some controversy,[5] single β-amino acids have since been incorporated into naturally occurring peptides to improve pharmacological properties.[6–10] The importance of β- and γ-amino acid containing compounds is further enhanced by their widespread occurrence in natural products of biological activity, originating from plants (e.g. the taxol side chain),[11] microorganisms (e.g. the enediyne chromophore of C-1027),[12] and marine organisms (often macrocyclic peptidic compounds[13]). Finally, simple β- and γ-amino acids also play a role in the metabolism of mammalians.[14]

Recently, we and others have shown that β- and γ-peptides, that is, oligomers of β- or γ-amino acids, form surprisingly stable secondary structures.[15–18] Gellman et al. have found that oligomers of cyclic β-amino acids, such as *trans*-2-aminocyclopentane- and *trans*-2-aminocyclohexane carboxylic acid, adopt helical conformations in solution and in the solid state.[19] Simultaneously, our group demonstrated that peptides composed of simple acyclic β-amino acids, containing the side chains of natural α-amino acids, form stable secondary structures.[20–22] To date, all major secondary structural motifs found in natural α-peptides, that is, helices,[23–27] parallel and antiparallel pleated sheets,[28] and hairpins[28] have been found for β-peptides of this latter type. Furthermore, β-peptides have been shown to form other structures, not commonly found in nature, including tubular stackings,[29, 30] ribbons,[31] and alternative helices.[32, 33] Analogously, our group[34, 35] and the group of Hanessian[36] have revealed that γ-peptides, built of open-chain γ-amino acid residues, also may adopt helical conformations.

One distinct characteristic of β- and γ-peptides is their ability to form the stable secondary structures with as few as four amino acid residues. This is in sharp contrast to α-peptides, where much longer sequences are required to obtain stable secondary structures. The high stability and predictability of β-peptidic conformations renders the β- and γ-peptides attractive backbones for drug design. β-Peptides may be used as rigid scaffolds to place functional groups in well-defined positions, for example, for interaction with a receptor. One such application

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has already been successfully demonstrated with β-tetrapeptides mimicking the α-tetradecapeptide somatostatin.[37–39]

Apart from their structural stability, β-peptides display a remarkable stability toward enzymatic degradation, as reported by our group.[21, 40, 41] The objective of the present study was to substantiate the preliminary results by testing a larger variety of enzymes and of β-peptides to be able to draw more general conclusions about the stability of β-peptides toward proteolytic enzymes. Thus, we have now investigated the proteolytic stability of β-peptides with functionalized side chains, with different substitution patterns, and with opposite configurations (i.e. D instead of L) at some, or at all stereogenic centers. The proteolytic stability of γ-peptides was also investigated for the first time. Furthermore, we examined whether β- and γ-peptides may have inhibitory activity toward chymotrypsin.

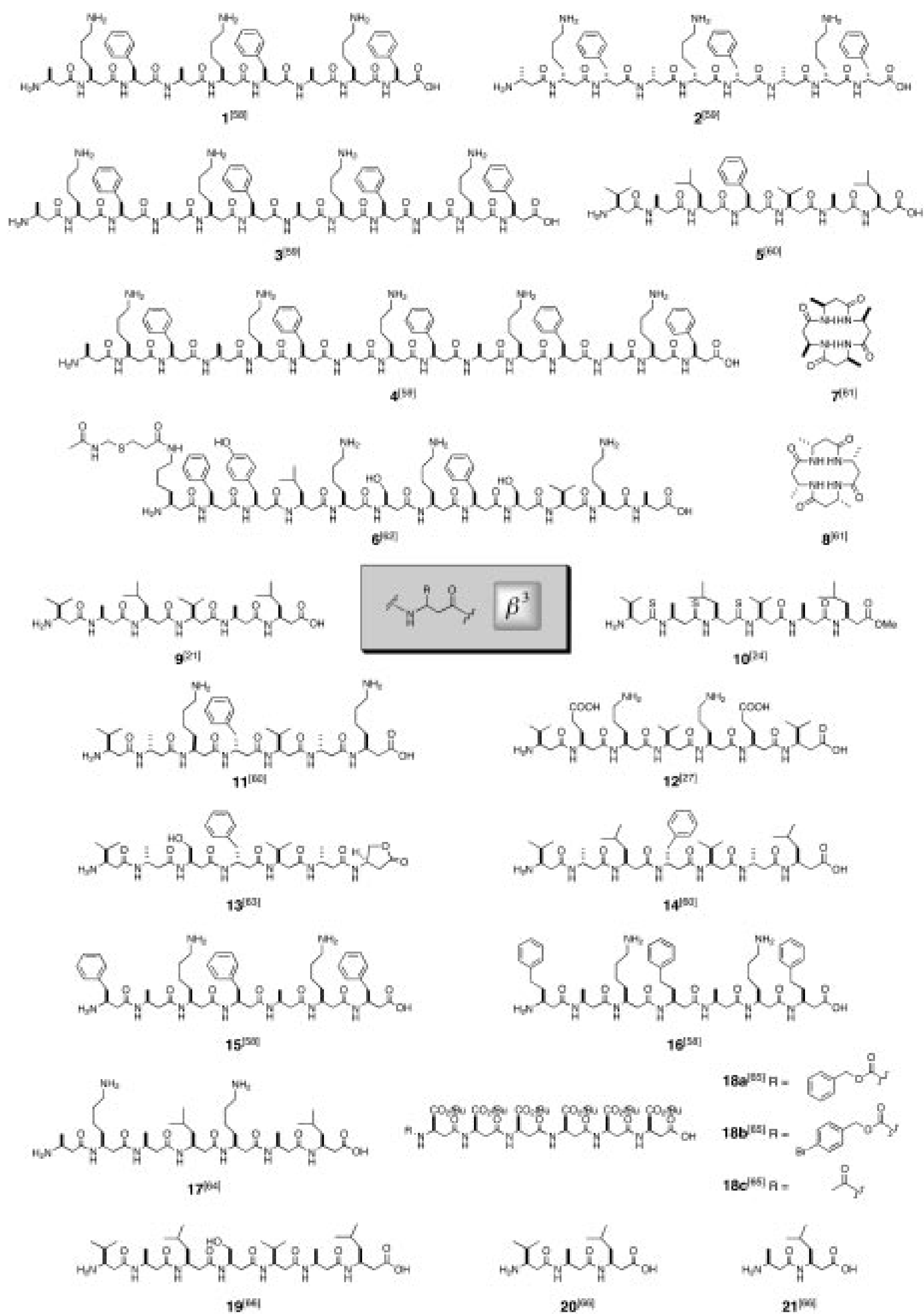
Choice of peptidases

The enzymes included in this study are representatives of the major classes of peptidases, for example, serine and aspartic peptidases and metallopeptidases, as well as of different biologically relevant sources, that is, bacteria, fungi and eukaryotes. Our choice is rationalized by a short description of the enzymes and their specificities in the following paragraph.[42, 43]

Pronase (EC 3.4.24.4) is a commercially available mixture of extracellular enzymes derived from the K-1 strain of the fungus *Streptomyces griseus*, and consists of both endo- and exopeptidases. The extract is known to contain at least three serine peptidases, that is, *Streptomyces griseus* trypsin, streptogrisin A and B, and the metallopeptidase *Streptomyces griseus* aminopeptidase. Pronase cleaves almost any peptide bond, as demonstrated by its ability to yield a large amount of free amino acids in the digestion of protein substrates.[44] Proteinase K (EC 3.4.21.64) is a serine peptidase from the mould *Tritirachium album*. It acts as an endopeptidase, which, unlike the mammalian proteolytic enzymes, exhibits a marked lack of selectivity toward peptide substrates. It splits peptide bonds of amino acids preferably at hydrophobic side chains.[45] Pepsin A (EC 3.4.23.1) is an aspartic peptidase from hog stomach. It is the principal protease of the stomach in higher organisms, and is characterized as an endopeptidase with low substrate specificity. The pH range of peptide hydrolysis ranges from 1 to 6, with an optimum efficiency near pH 3.5.[46] Chymotrypsin (EC 3.4.21.1) from bovine pancreas, elastase (EC 3.4.21.36) and trypsin (EC 3.4.21.4) from hog pancreas are all serine peptidases of the clan SA. All the proteolytic enzymes in this clan are endopeptidases. Besides their presence in eukaryotes, these enzymes are also known from RNA viruses and bacteria.[42] Chymotrypsin specifically hydrolyzes the amide bonds formed by the carboxy groups of Tyr, Phe, Trp, and Leu. Various ester and amide derivatives of the chymotrypsin-specific amino acids are also hydrolyzed by the enzyme. Elastase shows a marked primary specificity for nonbulky residues such as Ala, Ser, Gly, and Val in P1 (for a description of binding site nomenclature, see the Discussion section), while trypsin prefers to cleave amide substrates following the basic P1 residues Arg and Lys. Carboxypeptidase A (EC 3.4.17.1) is a zinc-dependent metallopeptidase isolated from bovine pancreas. It is an exopeptidase and cleaves the C-terminal peptide or ester bond of peptides or depsipeptides that have a free C-terminal carboxy group. Acylated amino- and hydroxycarboxylic acids are also substrates. Although the specificity of the enzyme is low, the rate of peptide substrate hydrolysis is enhanced when the side chain is aromatic or branched aliphatic.[47] Leucyl aminopeptidase (EC 3.4.11.1) from porcine kidney is also a zinc-dependent metallopeptidase. It is an exopeptidase that, despite what its name implies, hydrolyzes all compounds with an N-terminal L-amino acid (or glycine); however, compounds that have an L-leucyl residue at the N-terminal position are preferred.[48] Proteinase (EC 3.4.21.62) from *Bacillus subtilis var. biotecus A*, also known as Subtilisin Carlsberg or Subtilo peptidase A, is an extracellular serine endoproteinase. This enzyme belongs to the class of subtilisins which hydrolyze proteins with low specificity for substrates with peptide bonds, although with a preference for a large and uncharged residue in P1.[49] Peptidase from porcine intestinal mucosa is a commercially available nonspecific mixture containing general proteolytic and aminopeptidase activity. Pronase E (EC 3.4.24.31) from *Streptomyces griseus*, also known as mycolysin, is a single enzyme isolated from the pronase mixture described above.[50] It is also a zinc-dependent metalloendopeptidase with unusually nonspecific proteolytic activity; however, hydrophobic residues in P1′ are favored. Penicillin amidase (EC 3.5.1.11) from *Escherichia coli* is a hydrolase acting on carbon – nitrogen bonds other than peptide bonds. The enzyme catalyzes hydrolytic cleavage of the side chain of penicillins to produce the core structure, 6-aminopenicillanic acid, and the corresponding carboxylate.[51] β-Lactamase (EC 3.5.2.6) from *Enterobacter cloacae* is a mixture of enzymes with varying specificity for hydrolyzing β-lactams; some act more rapidly on penicillins, some more rapidly on cephalosporins. Generally, these enzymes catalyze the hydrolytic opening of the β-lactam ring to produce a substituted β-amino acid as product.[52] Amidase (EC 3.5.1.4) from *Pseudomonas aeruginosa* is a hydrolase, also acting on carbon – nitrogen bonds other than peptide bonds. It hydrolyzes monocarboxylic acid amides to ammonia and the corresponding free carboxylic acid.[53] Eukaryotic 20 S proteasome, isolated from human erythrocytes,[54] is a multicatalytic endopeptidase complex responsible for intracellular proteolysis.[55] The 20 S proteasome is composed of 28 subunits arranged in four rings of seven subunits. The outer rings are composed of α subunits, but the β subunits forming the inner rings are responsible for peptidase activity. In eukaryotic organisms there are up to seven different types of β subunits, three of which may carry the N-terminal threonine residues that are the nucleophiles in catalysis, and they show different specificities. The whole enzyme complex is thus characterized by an unusually low substrate specificity for the cleavage of peptide bonds.[56]

Choice of β- and γ-peptides

In the present study, we examined the 23 β-peptidic compounds (**1** – **21**, consisting of β^3-amino acids) shown in Scheme 1.[21, 24, 27, 58–66] Of these, **1** – **6**, **9** – **10**, **12**, and **15** – **16**



Scheme 1. *The all-β³-peptides used in this study. Numbers in square brackets refer to the references in which the preparation of the compounds has first been described.*

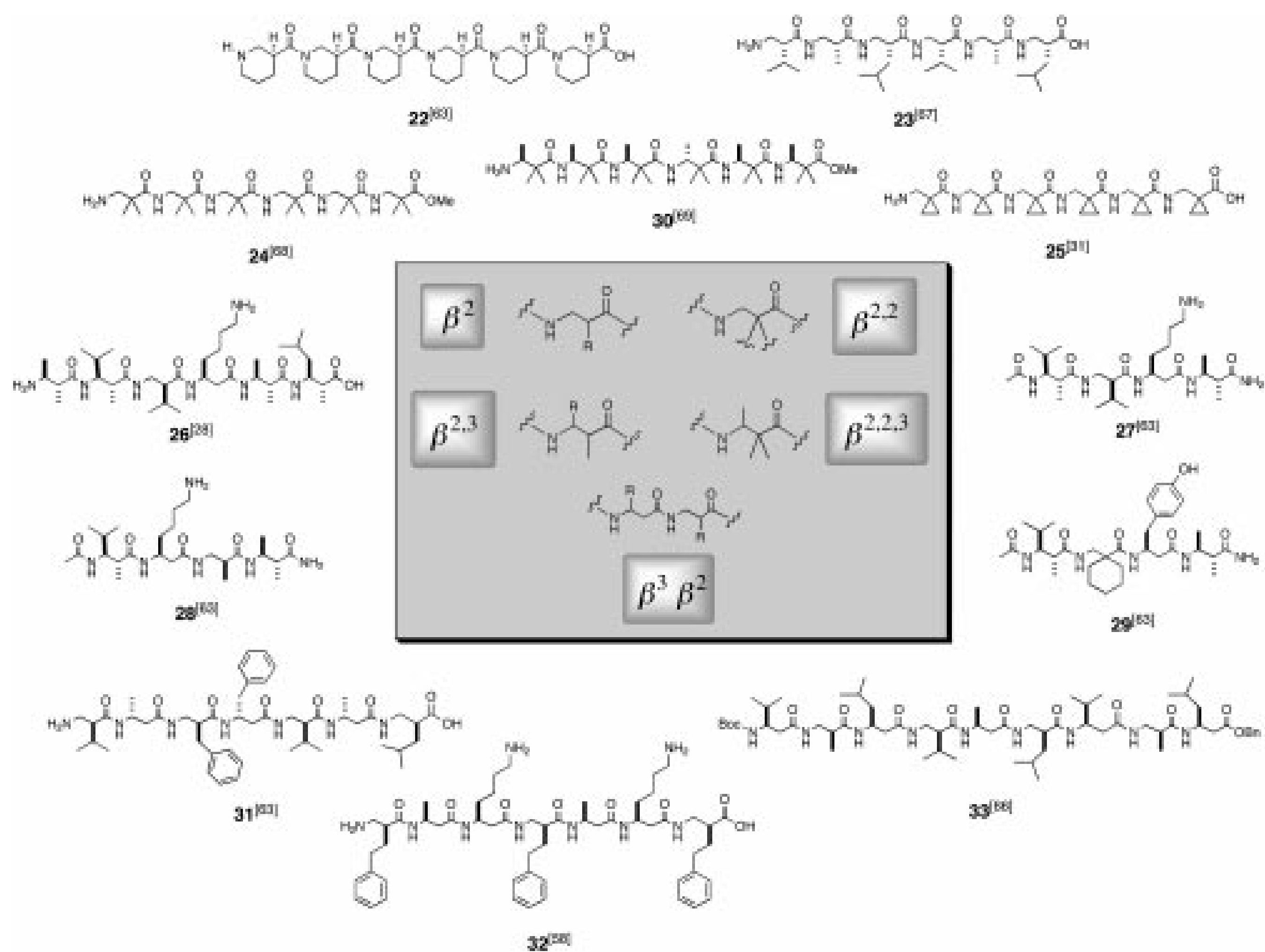
have been shown by NMR spectroscopy to form helical structures, or they display the circular dichroism (CD) spectra typical of the 3_{14}-helical structure. The molecules **7** and **8** form tubular arrangements in the solid state, so called "nanotubes";[57] while the secondary structures of **11**, **13** – **14**, and **18 a** – **c** are still unknown. Interestingly, compound **6** undergoes a drastic change of secondary structure upon change of solvent (MeOH vs. H_2O).

Additionally, the 12 compounds containing β^2 substituents depicted in Scheme 2 were tested.[28, 31, 58, 63, 66–69] In this class of compounds, a variety of secondary structures can be found. For example, **26** – **29** form a hairpin motif, **23** forms a 3_{14}-helical structure, while **25** forms a pleated ribbon not found in natural systems. Of the γ-peptides **34** – **37** (Scheme 3) used in this study,[70] only the $\gamma^{2,3,4}$-peptide **37** has so far been shown to form the 2.6_{14}-helix known from previous studies on γ^4-peptides. Nevertheless, analogues of the γ-tripeptide **36**, with a larger chain length, were also found to form helical structures.[34]

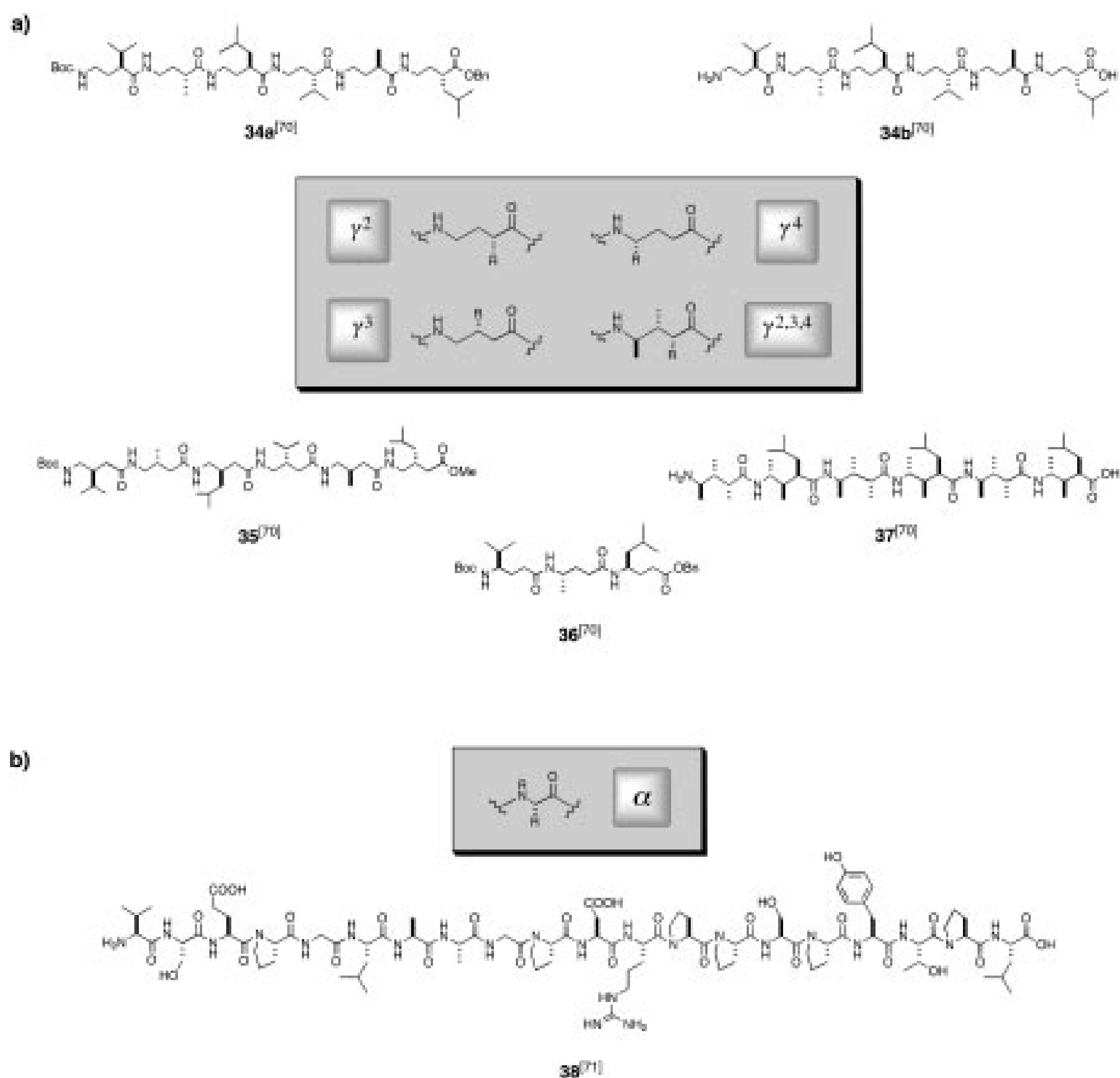
Results

Degradation studies

To gain insight into the activity of proteolytic enzymes toward β- and γ-peptides, we first chose to investigate degradation of some selected peptides with a wide variety of commercially available enzymes. Degradation studies were based on a method suitable for HPLC analysis as this allowed us to analyze a large number of β- and γ-peptides lacking special chromophoric groups. Furthermore, this procedure would enable us to isolate possible degradation products directly and analyze them by mass spectrometry. All enzymes were used in high specific concentrations, such that an α-peptide should be degraded within 10 min. As a standard substrate to verify the enzymatic activities we chose α-peptide **38** (Scheme 3), which is the C-terminal part of the SR-BII (scavenger receptor, class B, type II) protein.[71] The activity of penicillin-degrading enzymes was, however, tested against benzyl penicillin. All test substrates were entirely degraded within a maximum period of time of 60 min as shown by disappearance of the substrate peak and occurrence



Scheme 2. β-Peptides containing β²-substituted amino acids. Numbers in square brackets refer to the references in which the preparation of the compounds has first been described.



Scheme 3. *a) γ-Peptides of various substitution patterns. Numbers in square brackets refer to the references in which the preparation of the compounds has first been described. b) Molecular structure of the α-peptide used as test substrate.*

of smaller fragments in the corresponding HPLC traces (Figure 1 a). To safely prove the assumed stability of β- and γ-peptides we even chose more drastic conditions by incubating the peptides with highly concentrated solutions of enzymes for 48 h. Due to their hydrophobic nature and their resulting poor solubility in aqueous media, some peptides had to be dissolved by addition of DMSO. In most cases the amount of added DMSO could be limited to 20% except for γ-peptide **34 a**, and the cyclic tetrapeptides **7** and **8** (known to form tubular aggregates involving an infinite network of hydrogen bonds in pleated sheets). Nevertheless, DMSO addition, even in the high concentrations used in this study, did not have a significant impact on the activity of the enzymes, as verified by degradation of α-peptide **38** with concentrations of DMSO ranging from 10 to 50%. In addition, DMSO is known to disrupt intramolecular hydrogen bonding and is therefore expected to facilitate enzymatic hydrolysis by presenting an unfolded structure of the peptide to the enzyme.

In addition to α-peptide **38** we selected the β^3-hexa-, hepta-, and dodecapeptides **9**, **19** and **3**, respectively, the short β^3-peptides **20** and **21**, the β^2-peptide **23**, and the γ^2-hexapeptide **34 b** for an investigation with all enzymes mentioned above. The outcome of this first test series is summarized in Table 1. The results are evidence for the exceptional stability of β- and γ-peptides, as none of the peptides **3**, **9**, **19**, **20**, **21**, **23**, and **34 b** was digested after 48 h of incubation, whereas α-peptide **38** was entirely degraded within 1 h.

Nevertheless, these conclusions are based on the incubation of just a limited number of β- and γ-peptides, neglecting the influence of substituents and functional groups. As these factors are expected to influence the properties of β- and γ-peptides (stability and secondary structures), which have not yet been

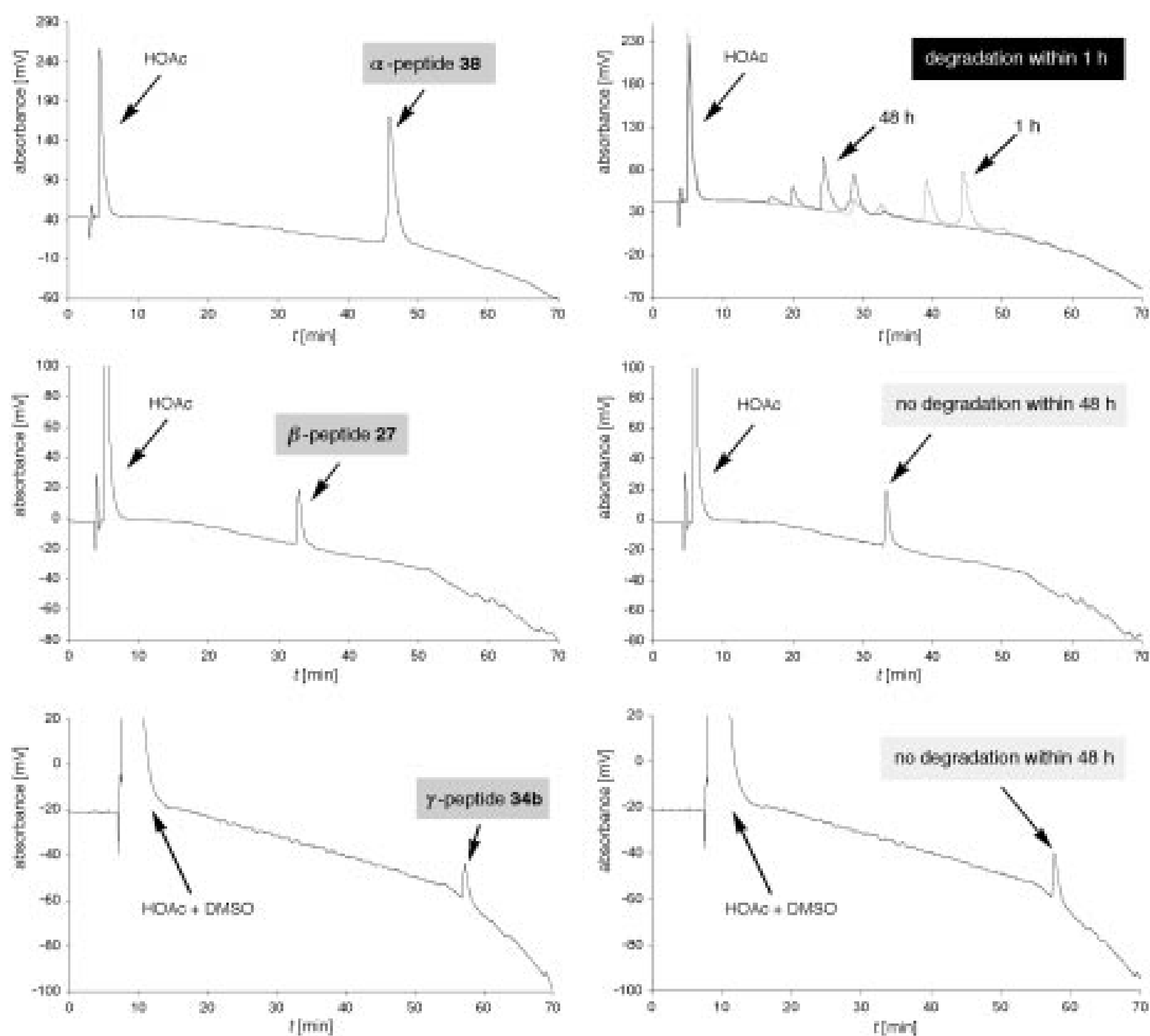


Figure 1. *HPLC analysis of the enzymatic degradation solutions of α-peptide **38**, β-peptide **27**, and γ-peptide **34b**.*

Table 1. *Treatment of selected β- and γ-peptides with various enzymes.*[a]

Entry	Enzyme	Origin of the enzyme	**38** (α)	**9** (β^3)	**23** (β^2)	**19** (β^3)	**20** (β^3)	**21** (β^3)	**3** (β^3)	**34b**[b] (γ)
1	pronase	*Streptomyces griseus*	+		–	–	–	–	–	–
2	proteinase K	*Tritirachium album*	+		–	–	–	–	–	–
3	pepsin	hog stomach	+	–	–	–	–	–	–	–
4	chymotrypsin	bovine pancreas	+	–	–	–	–	–	–	–
5	elastase	hog pancreas	+	–	–	–	–	–	–	–
6	trypsin	hog pancreas	+	–	–	–	–	–	–	–
7	carboxypeptidase A	bovine pancreas	+	–	–	–	–	–	–	–
8	leucyl aminopeptidase	porcine kidney	+	–	–	–	–	–		–
9	proteinase	*Bacillus subtilis var. biotecus* A	+			–	–	–	–	–
10	peptidase	porcine intestinal mucosa	+			–	–	–	–	–
11	pronase E	*Streptomyces griseus*	+			–	–	–	–	–
12	penicillin amidase	*Escherichia coli*			–				–	–
13	β-lactamase	*Enterobacter cloacae*			–				–	–
14	amidase	*Pseudomonas aeruginosa*	–		–				–	–
15	20 S proteasome	human erythrocytes							–[c]	

[a] The measurements with β-peptides **9** and **23** in entries 3 – 8 have been described previously.[40] A + sign indicates that degradation takes place, – that no degradation is detectable. [b] This peptide had to be dissolved in a mixture of PBS buffer and DMSO. [c] Nonamer **1** containing the triades β^3-HAla-β^3-HLys-β^3-HPhe was tested instead of **3**.[41]

elucidated, we focussed on structural diversity of the substrates in a second round of our degradation studies.

As one of the main objectives of the present study was to investigate the large structural variety of the peptides depicted in Schemes 1 – 3, we selected just a limited number of enzymes for the succeeding studies. Pronase and proteinase K were selected due to their high activity and broad substrate specificity. Furthermore, pronase appeared especially suitable since it consists of a mixture of several exo- and endopeptidases containing both serine- and metallopeptidases. After having learned about the stability of β-peptides toward ordinary peptidases, we also tested other hydrolytic enzymes not specifically recognizing normal peptide bonds. Penicillin amidase and β-lactamase were thus selected for tests since these two enzymes recognize the β-amino carbonyl fragment found in penicillin and cephalosporin derivatives, and hence could also have recognized the β-amino acid motif of β-peptides. Additionally, we included amidase in this study, hoping that this enzyme would recognize the C-terminal amide group present in some of the investigated compounds (i.e. **27** – **29**).

We first investigated the incubation of β^3-substituted peptides with the selected enzymes (Table 2). The result is unambiguous, neither the pure aliphatic peptides (**5**, **7** – **10**, **14**, and **18**) nor the peptides containing hydrophilic side chains (**1**, **2** – **4**, **6**, **11** – **13**, **15** – **17**) were degraded.

As seen from the data presented in Table 3, β-peptides containing substituents in the 2-position are absolutely stable toward enzymatic degradation, and this does not depend on configuration or substitution pattern. In addition to peptide **22**, which is derived from (*S*)-nipecotic acid, the β^2-, $\beta^{2,2}$-, $\beta^{2,3}$-, $\beta^{2,2,3}$-, and $\beta^2\beta^3$-substituted peptides **23** – **33** were tested against pronase, proteinase K, penicillin amidase, and β-lactamase. Likewise, peptides **27** – **29**, containing a C-terminal amide group, were tested against amidase, however, without any observable cleavage.

For the first time, γ-peptides were included in an enzymatic degradation study. To extend the initial stability test of γ^2-substituted peptide **34b** with a variety of enzymes, γ-peptides **34a** and **35** – **37** containing aliphatic γ^2-, γ^3-, γ^4-, and $\gamma^{2,3,4}$-substituents were also incubated with the enzymes chosen in our tests. The outcome of these experiments was that no degradation was observed (Table 4).

Table 2. *Degradation experiments with β³-peptides, using various proteolytic enzymes.*[a]

Peptide	DMSO addition[b]	Enzyme			
		pronase	proteinase K	penicillin amidase	β-lactamase
1	–	×	×	×	×
2	–	×	×	×	×
4	–	×	×	×	×
5	–	×	×		
6	–	×	×	×	×
7	50	×	×	×	×
8	50	×	×	×	×
10	20	×	×	×	×
11	–	×	×	×	×
12	–	×	×	×	×
13	–	×	×		
14	–	×			
15	–	×	×		
16	–	×	×		
17	–	×	×		
18a	20	×	×	×	×
18b	20	×	×	×	×
18c	20	×	×	×	×

[a] The peptides were incubated as aqueous or DMSO-containing aqueous solutions. No degradation was detectable in any of these experiments (HPLC analysis). × means that the test was performed, with no degradation detectable. [b] The effect of DMSO addition was neglectable, as the corresponding test substrate, α-peptide **38**, was still degraded after 1 h with 10% and 50% DMSO added to the buffer solution.

Table 3. *Treatment of β²-substituted β-peptides with various proteolytic enzymes.*[a]

Peptide	DMSO addition	Enzyme				
		pronase	proteinase K	amidase	penicillin amidase	β-lactamase
22	–	×	×	×	×	×
24	10	×	×		×	×
25	–	×	×		×	×
26	–	×	×		×	×
27	–	×	×	×	×	×
28	–	×	×	×	×	×
29	–	×	×	×	×	×
30	10	×				
31	–	×	×			×
32	20	×	×			
33	–	×	×		×	×

[a] The peptides were incubated as aqueous or DMSO-containing aqueous solutions. No detectable degradation was observed in any of the cases. × means that the test was performed, with no degradation detectable.

Table 4. *Treatment of γ-peptides with a series of proteolytic enzymes.*[a]

Peptide	DMSO addition	Enzyme			
		pronase	proteinase K	penicillin amidase	β-lactamase
34a	40	no degradation			
34b	20	no degradation			
35	20	no degradation			
36	20	no degradation			
37	–	no degradation			

[a] The peptides were incubated as aqueous or DMSO-containing aqueous solutions.

Inhibition studies

To find out whether the enzymes used were still active after prolonged incubation with β- and γ-peptides, that is, whether the homologues of α-peptides have a deactivating effect on these enzymes or not, we tested their enzymatic activities toward chromophoric standard substrates after 48 hours: A set of enzymes (elastase, proteinase K, proteinase, leucin aminopeptidase, carboxypeptidase A, and chymotrypsin) were incubated with the β-peptides **2**, **3**, **19**, **22**, **25**, **27** – **29** for 48 hours and tested for activity by adding the corresponding test substrate (see Experimental Section for details). The result was that all enzymes were still active after incubation.

A quantitative study on the inhibitory potential of some β- and γ-peptides against chymotrypsin was also performed (following Geiger's protocol).[72] Buffered solutions of chymotrypsin were incubated with β- and γ-peptides **2**, **3**, **19**, **25**, **27**, **29**, and **34b** for 15 min at 25 °C and then treated with a solution of the test substrate (Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide). The release of *p*-nitroaniline was measured at 405 nm by using a thermostated UV spectrophotometer. The results are presented in Table 5.

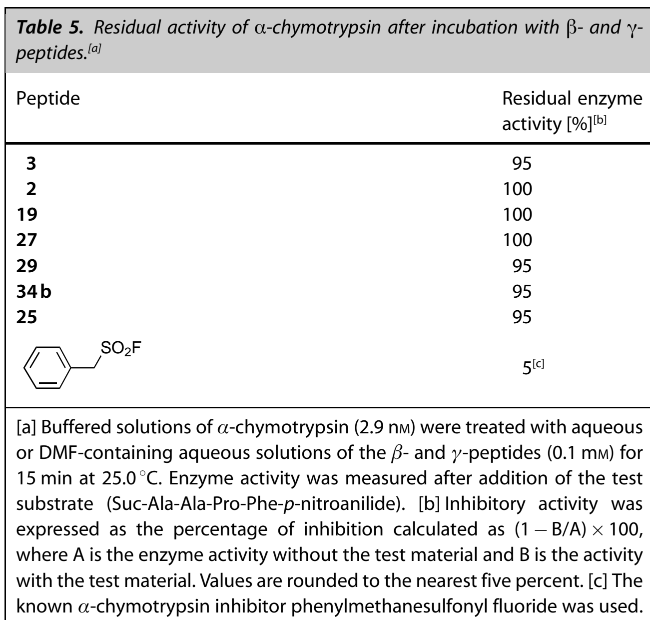


Table 5. Residual activity of α-chymotrypsin after incubation with β- and γ-peptides.[a]

Peptide	Residual enzyme activity [%][b]
3	95
2	100
19	100
27	100
29	95
34b	95
25	95
(phenylmethanesulfonyl fluoride, SO_2F)	5[c]

[a] Buffered solutions of α-chymotrypsin (2.9 nM) were treated with aqueous or DMF-containing aqueous solutions of the β- and γ-peptides (0.1 mM) for 15 min at 25.0 °C. Enzyme activity was measured after addition of the test substrate (Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide). [b] Inhibitory activity was expressed as the percentage of inhibition calculated as $(1 - B/A) \times 100$, where A is the enzyme activity without the test material and B is the activity with the test material. Values are rounded to the nearest five percent. [c] The known α-chymotrypsin inhibitor phenylmethanesulfonyl fluoride was used.

No significant inhibition of chymotrypsin by any of the tested β- or γ-peptides could be detected, even at a peptide concentration of 0.1 mM (corresponding to a peptide/enzyme molar ratio of $>10^6$).

Discussion

Peptides composed of β- and γ-amino acids evidently have a high resistance toward proteolytic degradation, as demonstrated in this and other studies from our laboratory. Although no degradation was observed, it is interesting to make some comments about the outcomes of these studies. As evident from the results in Table 1, the mode of action and specificity of all the different enzymes investigated did not have any impact on the degradability of the β- and γ-peptides **3**, **9**, **19**, **20**, **21**, **23**, and **34b**. Furthermore, the following conclusions can be drawn from the data in Table 2: i) The configuration of the chirality centers does not affect the outcome of the degradation experiments, as seen from the enantiomeric pairs **1**/**2** and **7**/**8**, and from the peptides with alternating configuration **11**, **13**, and **14**. ii) Cyclization of the β-peptide, as in **7** and **8**, or iii) sulfur substitution as in the thiopeptide **10** did not facilitate proteolysis. iv) Peptides **18a–c**, containing the only proteinogenic β-amino acid, aspartic acid, were also not recognized by any of the proteolytic enzymes! Incorporation of β^2-amino acids leads to peptides with the side chains positioned next to the carbonyl group of the amide bond. Neither the *all*-β^2-peptides **22–25**, nor the β-peptides **26–33** consisting of $\beta^{2,2}$-, $\beta^{2,3}$-, $\beta^{2,2,3}$-, or $\beta^2\beta^3$-substituted amino acids were degraded (Table 3); note that **31–33** contain two to four amide bonds flanked by two side chains, just like natural α-peptides. The observed stability of γ-peptides toward the proteolytic enzymes (Table 2 and Table 3) is of considerable importance as it suggests that also γ-peptides may be suitable for pharmaceutical applications.

The lack of any observable degradation or inhibition substantiates our suggestion that interactions between β- and γ-peptides, on the one hand, and natural proteins such as enzymes and receptors, on the other hand, can only be mediated through side chain functionalities. The interaction between proteolytic enzymes and their substrates may be described by using the nomenclature of Schechter and Berger.[73] The binding site for a polypeptide substrate on a protease has different subsites; each subsite interacts with one amino acid residue of the substrate. As illustrated in Figure 2a, the amino acid residues on the N-terminal side of the bond to be cleaved are numbered P1, P2, P3 ... counting outward, while the residues on the C-terminal side of the bond to be cleaved are numbered P1′, P2′, P3′ ...; the complementary subsites on the protease are termed S3, S2, S1, S1′, S2′, S3′.

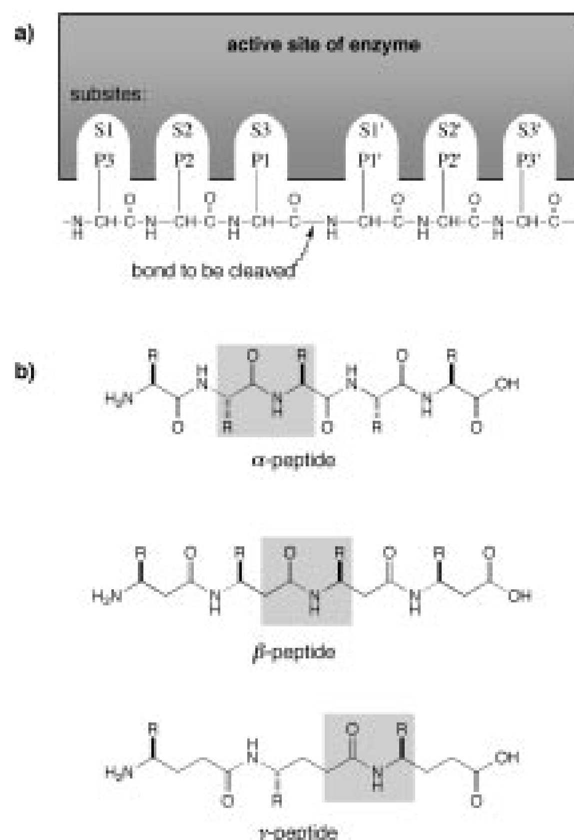


Figure 2. a) Schematic representation of the binding of a substrate to the binding pockets of a proteolytic enzyme according to the nomenclature of Schechter and Berger.[73] b) Comparison of an extended conformation of α-, β-, and γ-peptides, highlighting the substitution pattern around the amide bonds.

The substrate binds to the enzyme in an extended conformation through hydrophobic, electrostatic, and hydrogen-bonding interactions.[74] A comparison of the extended conformations of α-, β-, and γ-peptides reveals that there are large differences, indeed (Figure 2b). The side chains in β- and γ-peptides have different spacing, as compared to natural α-peptides, and all amide bonds of β-peptides have the same orientation. Furthermore, none of the investigated peptides has the side chains next to amide bonds in the same spatial arrangement as do natural α-peptides. It will be interesting to see whether it is possible to introduce by design an amide bond, cleavable by an enzyme, in a β-peptide by choosing suitable side chains in the right position *and* with the right configuration. Considering the huge structural differences, it is maybe not surprising, after all, that the worlds of β- and γ-peptides appear to be orthogonal to the world of α-peptides, at least when backbone interactions are important. The amide bonds in α- and β-peptidic structures may interact through single hydrogen bonds; however, multiple hydrogen bonds are not possible due to the different spacing of the interacting groups. The data collected to date suggest that communication between the distinct worlds has so far only been mediated through the side chains, as testified by the somatostatin example mentioned in the Introduction.[37–39]

Conclusion

This study has demonstrated the outstanding stability of β- and γ-peptides toward proteolytic enzymes, while other studies have demonstrated the potential of β-peptides as peptidomimetics in in vivo experiments. Although they are promising candidates, there is still no guarantee that β- and γ-peptides of medicinal interest will have good bioavailability. Absorption barriers and hepatobiliary excretion mechanisms may still compromise the therapeutic potential of this class of compounds as peptidomimetic drugs, thereby subjecting them to acute or chronic intravenous administration requirements. Studies aiming at answering these questions are currently underway, using radioactively labeled compounds.[79]

Experimental Section

Reagents and enzymes: The following reagents and enzymes were obtained commercially: $CaCl_2 \cdot H_2O$ (Merck), Trizma Base (tris[hydroxymethyl]aminomethane, Sigma), Triton X-100 (Sigma), Suc-$(Ala)_3$-4-nitroanilide (Bachem), Suc-$(Ala)_2$-Pro-Phe-4-nitroanilide (Bachem), *N*-(4-methoxyphenylazoformyl)-Phe-OH potassium salt (Bachem), L-leucine-4-nitroanilide (Fluka), fuming HCl 37% (Merck), acetic acid (Scharlau), phenylmethanesulfonyl fluoride (Fluka), benzyl penicillin (Fluka), cephalosporin A (Fluka), NaCl (J. T. Baker), $Na_2HPO_4 \cdot 2H_2O$ (Fluka), $NaH_2PO_4 \cdot H_2O$ (FisherChemical), NaOH pellets (Siegfried Synopharm). All peptides were used as trifluoroacetic acid (TFA) salts as obtained after lyophilization. Water used for preparing buffers and other solutions was of Nanopure quality.

Pronase (EC 3.4.24.4) from *Streptomyces griseus* (Fluka), lyophilized powder, stock solution in PBS buffer (pH 7.5, 0.01 M). Pronase E (EC 3.4.24.4) from *Streptomyces griseus* (Fluka), lyophilized powder, stock solution in PBS buffer (pH 7.5, 0.01 M). Proteinase K (EC 3.4.21.64) from *Tritirachium album* (Fluka), suspension in 10 mM Tris-HCl, pH 7.5, 40% glycerol, 1 mM Ca-acetate; stock solution in PBS buffer (pH 7.5, 0.01 M). Amidase (EC 3.5.1.4) from *Pseudomonas aeruginosa* (Sigma), solution in 50% glycerol and phosphate buffer, stock solution in PBS buffer (pH 7.5, 0.01 M). Penicillin amidase (EC 3.5.1.11) from *Escherichia coli* (Sigma), solution in 0.1 M, potassium phosphate buffer (pH 7.5), stock solution in PBS buffer (pH 7.5, 0.01 M). β-Lactamase (EC 3.5.2.6) from *Enterobacter cloacae* (Fluka), lyophilized powder, stock solution in PBS buffer (pH 7.0, 0.01 M). Carboxypeptidase A (EC 3.4.17.1) from bovine pancreas (Fluka), milky suspension, stock solution in PBS buffer (pH 7.5, 0.01 M). α-Chymotrypsin (EC 3.4.21.1) from bovine pancreas (Fluka), lyophilized powder, stock solution in PBS buffer (pH 8.0, 0.01 M). Elastase (EC 3.4.21.36) from hog pancreas (Fluka), lyophilized powder, stock solution in PBS buffer (pH 7.5, 0.01 M). Leucyl aminopeptidase (EC 3.4.11.1) from porcine kidney (Sigma); chromatographically purified suspension in 2.9 M $(NH_4)_2SO_4$, 0.1 M Tris, 5 mM $MgCl_2$ solution, pH 8.0; stock solution in PBS buffer (pH 7.2, 0.01 M). Pepsin (EC 3.4.23.1) from hog stomach (Fluka), lyophilized powder, stock solution in AcOH (pH 2.1, 1.74 M). Peptidase (no EC number, EEC 232-875-1) from porcine intestinal mucosa (Sigma), lyophilized powder, stock solution in PBS buffer (pH 7.1, 0.01 M). Proteinase (EC 3.4.21.11) from *Bacillus subtilis var. biotecus A* (Fluka), lyophilized powder, stock solution in PBS buffer (pH 7.5, 0.01 M). Trypsin (EC 3.4.21.4) from hog pancreas (Fluka), lyophilized powder, stock solution in PBS buffer (pH 7.5, 0.01 M). Purified 20S proteasome was obtained from human erythrocytes.[54]

Devices: HPLC analysis: Analytical HPLC was performed on a Knauer HPLC system (pump type 64, EuroChrom 2000 integration package, degasser, UV detector (variable-wavelength monitor)), Macherey-Nagel C_{18} column (Nucleosil 100-5 C_{18} (250×4 mm)). The samples were directly injected from the reaction mixture, using a Spark-Midas 830 auto sampler.

pH measurements: The buffer solutions were pH-tested at 25 °C with a digital Metrohm 632 pH meter that had been referenced to pH 7.00 with a buffer standard solution (color-coded, Fluka).

UV spectroscopy: Absorbance measurements were made on a Perkin – Elmer UV/Vis spectrophotometer (Lambda 40) which was controlled by UV Winlab operating software. The 1-cm quartz cells (1.5 mL) were held during the assays in a themostated cell block that was controlled by a Perkin – Elmer PTP-6 Peltier System (set at 25.0 ± 0.2 °C). The substrate solution was stored during use in a water bath thermoregulated at 25 °C by a Haake FS2 water bath thermostat. The residual activity assay was performed on a SPECTRAmax 250 Microplate spectrophotometer using SOFTMAX PRO software for data analysis.

Assays: Degradation experiments: For each degradation experiment stock solutions of the peptidic substrate, the enzymes, and suitable test substrates for testing the enzyme activities were made using phosphate-buffered saline (PBS) buffer solution. The experiments with pepsin were carried out in AcOH. For all enzymes, except for amidase, β-lactamase and penicillin amidase, α-peptide **38** was chosen as standard substrate. Benzyl penicillin and cephalosporin A were used to test the activity of β-lactamase and penicillin amidase. The enzyme concentrations of the stock solutions were selected such that the standard substrates (same concentration as β- and γ-peptides) were totally degraded after a maximum of 15 min. Concentrations of some of the enzymes (pronase, amidase, and penicillin amidase) were chosen to cleave the test substrate within 1 min, whereas the concentration of proteinase K allowed degradation of a standard substrate within 6 s to establish forcing conditions for the corresponding degradation experiments of β- and γ-peptides. PBS buffer solution (10 mM sodium phosphate, 0.14 M

NaCl) was prepared as follows: Solution A (4.75 mL of 0.2 M $NaH_2PO_4 \cdot H_2O$), solution B (20.25 mL of 0.2 M $Na_2HPO_4 \cdot H_2O$), NaCl (4.5 g) and H_2O (25 mL) were stirred for 15 min at RT and diluted tenfold with H_2O. The pH was adjusted either with NaOH (1 M) or with HCl (1 M) prior to use.

General procedure: A solution of the substrate (200 µg) in PBS buffer (pH 7.5, 0.01 M) or in acetic acid (1.74 M, pH 2.1), in the case of degradation experiments with pepsin and a solution of the required amount of enzyme, were mixed and incubated at 25 °C (β-lactamase, carboxypeptidase A, elastase, chymotrypsin, trypsin, leucyl aminopeptidase) or 37 °C (pronase, pronase E, proteinase K, penicillin amidase, amidase, proteinase, pepsin, peptidase) for 48 h. Degradation was stopped with conc. AcOH and buffer solution was added so that the total volume reached 150 µL. The resulting mixture was analyzed by reversed-phase HPLC. Detection was achieved by measurement of the UV absorption at 220 nm. A mixture of CH_3CN/H_2O (0.1 % TFA) was used as eluent. Gradients were 0% CH_3CN for 5 min, in 45 min to 35% CH_3CN, in 15 min to 70% CH_3CN and up to 90% CH_3CN in another 5 min. The flow rate for all separations was 1 mL min^{-1}.

Residual activity assay: The assay was carried out at a substrate concentration of 0.6 mM in PBS buffer solution (pH 7.5) in 96-well microtiter plates. The enzyme solutions (50 µL) were incubated with the corresponding β-peptides (50 µL, 0.1 M) for 48 h at 25 °C (elastase, leucin aminopeptidase, carboxypeptidase A and chymotrypsin) and at 37 °C (proteinase K, proteinase), respectively. Thereafter, test substrates (150 µL; Suc-$(Ala)_3$-*p*-nitroanilide[75] for elastase, proteinase K, and proteinase; L-leucine-*p*-nitroanilide[76] for leucine aminopeptidase; *N*-(4-methoxyphenylazoformyl)-Phe-OH potassium salt[77] for carboxypeptidase A; Suc-$(Ala)_2$-Pro-Phe-*p*-nitroanilide for chymotrypsin[78]) were added and kinetic measurements were performed at 405 nm (*p*-nitroanilide derivatives) and at 350 nm (*N*-(4-methoxyphenylazoformyl)-Phe-OH potassium salt).

Inhibition studies: Inhibition of α-chymotrypsin was determined in Tris buffer solution with Suc-$(Ala)_2$-Pro-Phe-4-nitroanilide as test substrate following an assay procedure described by Geiger.[72] Each measurement was carried out three times. The resulting data sets were fitted and the initial rate was calculated by the operating software. Tris buffer solution was prepared as follows: A) Trizma Base (12.2 g, 0.10 mol), Triton X-100 (0.5 g), $CaCl_2 \cdot 2H_2O$ (2.9 g, 0.02 mol) were dissolved in water (800 mL) in a 1000-mL volumetric flask; 1 M HCl was added to reach pH 7.8 and the solution was diluted with water to a total volume of 1000 mL. B) Trizma Base (12.2 g, 0.10 mol) and $CaCl_2 \cdot 2H_2O$ (2.9 g, 0.02 mol) were dissolved in water (800 mL) in a 1000-mL volumetric flask. A 1 M HCl solution was added to reach pH 7.8 and the solution was diluted up to 1000 mL with water.

Substrate solution: *N*-Suc-$(Ala)_2$-Pro-Phe-4-nitroanilide (8.785 mg, 0.014 mmol, for inhibition studies with β-peptides; 7.472 mg, 0.012 mmol, for test experiments with the α-chymotrypsin inhibitor phenylmethanesulfonyl fluoride) was dissolved in buffer B (4.183 mL for β-peptides and 3.588 mL for phenylmethanesulfonyl fluoride, respectively) by sonication for 15 min.

β-Peptide and inhibitor solutions: β-Peptides **3** (812 µg), D-enantiomer **2** (536 µg), **19** (329 µg), and **27** (284 µg) were dissolved in buffer B (541 µL, 467 µL, 532 µL, and 570 µL, respectively), whereas β-peptides **29** (233 µg), **34b** (326 µg), **25** (455 µg), and phenylmethanesulfonyl fluoride (2.464 mg) were dissolved in DMF (505 µL, 537 µL, 969 µL, 20.20 mL, respectively).

Enzyme solution: A solution of Triton X-100 (0.05 g) and HCl (20.8 µL, 37%) was diluted to 100 mL with water. α-Chymotrypsin (239 µg) was dissolved in a sample of this solution (95.6 mL).

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[79] Note added in proof: The β-peptides **7**, **11**, **13**, and **27** have also been exposed to the PHB depolymerases A (PhaZ5), A (PhaZ7), and B (PhaZ2), enzymes which degrade, in an *endo* fashion, the biopolymer poly((*R*)-3-hydroxybutanoic acid), consisting of β-hydroxy-acid residues. No cleavage of a β-peptidic bond could be detected. The experiments were conducted in the laboratory of Professor D. Jendrossek (Universität Stuttgart, Germany) and at ETH (J. Frackenpohl).

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