

4-6-2009

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Recommended Citation

Ahmed S, Kaur K. The proteolytic stability and cytotoxicity studies of l-aspartic acid and l-diaminopropionic acid derived β -peptides and a mixed α/β -peptide. *Chem. Biol. Drug Des.* 2009;73(5):545-552. doi: 10.1111/j.1747-0285.2009.00803.x

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Comments

This is the accepted version of the following article:

Ahmed S, Kaur K. The proteolytic stability and cytotoxicity studies of l-aspartic acid and l-diaminopropionic acid derived β -peptides and a mixed α/β -peptide. *Chem. Biol. Drug Des.* 2009;73(5):545-552.

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The Proteolytic Stability and Cytotoxicity Studies of L-Aspartic Acid and L-Diaminopropionic Acid derived β -Peptides and a Mixed α/β -Peptide

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Keywords: β -peptides · α/β -peptides · proteolysis · enzymes · cytotoxicity

Abstract

The use of peptides as drugs in pharmaceutical applications is hindered by their susceptibility to proteolysis and therefore low bioavailability. β -Peptides that contain an additional methylene group in the backbone, are gaining recognition from a pharmaceutical stand point as they are considerably more resilient to proteolysis and metabolism. Recently we reported two new classes of β -peptides, β^3 - and β^2 -peptides derived from L-aspartic acid and L-diaminopropionic acid, respectively. Here we report the proteolytic stability of these β -peptidic compounds and a mixed α/β -peptide against three enzymes (pronase, trypsin, and elastase), as well as, human serum. The stability of these peptides was compared to an α -peptide. Peptides containing β -linkages were resistant to all conditions. The mixed α/β -peptide, however, exhibited proteolysis in the presence of trypsin and pronase but not elastase. The rate of degradation of the mixed α/β -peptide was slower than that would be expected for an α -peptide. In addition, these β -peptides were not toxic to HeLa and COS-1 cell lines as observed by MTT cytotoxicity assay. These results expand the scope of mixed α/β -peptides □containing β -amino acids or small β -peptide fragments as therapeutic peptides.

Introduction

Peptidomimetic β -peptides display remarkable stability against degradation by proteolytic enzymes (1-6). Hence, β -peptides with a wide variety of potential applications in medicinal chemistry have been synthesized, such as antibacterial agents (7, 8), virus entry inhibitors (9), and inhibitors of protein-protein interactions (10, 11). Such properties of β -peptides as medicinal agents stem from their ability to fold into stable secondary structures without the need for tertiary interactions in a relatively short oligomer (12). The proteolytic stability of β -peptides arises due to lack of any interaction between them and the *in vivo* enzymes. However, to be an ideal drug candidate, correct balance between the stability and the affinity for the biological receptor is highly desired.

Several strategies have been used to obtain appropriate balance between proteolytic stability, bioavailability, and biological activity, such as design of mixed peptides containing α - and β -amino acid residues or design of peptides where the backbone and the side chains together mimic the natural α -peptide (2, 5, 13). Efforts to modify the electronic environment by the introduction of electron withdrawing groups adjacent to the backbone carbonyl of a β -peptide bond failed to facilitate proteolytic degradation (14). Designing β -peptides, such as $N-(L)\beta^2hXaa-(L)\beta^3hXaa-C$, that resemble α -peptide with respect to the position and configuration of the side chain groups also did not make it susceptible to proteolytic degradation (2). We have recently reported two new classes of β -peptides, β^3 - and β^2 -peptides derived from L-aspartic acid (L-Asp) and L-diaminopropionic acid (L-Dap) monomers, respectively (15, 16). It is speculated that the β -peptides from L-Asp and L-Dap monomers may be susceptible to enzymatic cleavage due to the presence of an additional amide bond in the side chain. In addition, β^3 -peptide from L-Asp units contains an intrinsic L- α -amino acid (or α -linkage) in the sequence that may facilitate recognition and hydrolysis by peptidases. Finally, mixed α/β -peptides containing both α - and β -amino acid residues, where β -amino acids are derived from L-Asp, within a

peptide should allow achievement of a better balance between proteolytic stability and biological activity (3, 17). It is worth mentioning, however, that several bacterial enzymes that cleave β/β -peptide bond, such as β -peptidyl amino peptidases and poly(aspartic acid) hydrolases, have been identified (17, 18).

Accordingly, the proteolytic stability of four peptides **1-4** (Fig. 1) derived from L-Asp monomers (β^3 -hexapeptide **1**), L-Dap monomers (β^2 -hexapeptides **2** and **3**), and both α - and β^3 -amino acid monomers (mixed α/β^3 -peptide **4**) is compared with an α -hexapeptide **5**. The proteolytic stability of these representative peptides was tested against three enzymes, namely, pronase, trypsin, and elastase, as well as, against human serum. While β -peptides **1-3** were stable toward all the enzymes, mixed α/β -peptide **4** was completely degraded in 24 h by pronase and trypsin. The cytotoxicity of β -peptides **1** and **2** in HeLa and COS-1 cells is also discussed.

Fig. 1 - Chemical structures of peptides **1-5** studied herein. α -amino acids are shown in grey.

Materials and Methods

Solvents and reagents.

Rink amide MBHA resin (0.58 mmol/g), all amino acids, BOP, and HOBt were purchased from NovaBiochem (San Diego, CA). All other reagents were purchased from Sigma-Aldrich. All commercial reagents and solvents were used as received. Double distilled water was autoclaved and used for preparing buffers. Trypsin from porcine pancreas (lyophilized powder, 1,000-2,000 BAEE units/mg), pronase E from *Streptomyces griseus* (lyophilized powder, slightly brown, ~6 units/mg), and elastase from hog pancreas (EC 3.4.21.36) were purchased from Sigma-Aldrich. HeLa cells were

provided by the Campbell Laboratory (Department of Chemistry, University of Alberta), while COS-1 cells were obtained from the Suresh Laboratory (Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta). TAT₄₇₋₅₇ (YGRKKRRQRRR) peptide was purchased from GenScript Corporation (USA).

pH measurements of all buffer solutions were done at 25 °C with a digital SB20 Symphony VWR pH meter using a calibration buffer set (Fluka). Reversed phase (RP) HPLC analyses were carried out on a Waters (625 LC system) HPLC system using Vydac analytical C8 column (0.46 x 25 cm, 5 µm) using an auto-injector mode. Column effluent was monitored by UV detection at 220 nm. Mass spectra were recorded on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Voyager mass spectrometer (VoyagerTM Elite) or on a Waters micromass ZQ. Absorbance of the purple formazan product observed during MTT assay was measured using a VERSA max microplate reader (Molecular Devices). All the procedures regarding the cell culture maintenance and treatment of cells were carried out in a level II biosafety cabinet.

Peptide Synthesis

Synthesis of peptides **1** and **2** has been reported previously (15). Peptides **3-5** were synthesized similarly following stepwise manual Fmoc solid-phase synthesis.

β²-peptide 3. Fmoc/allyl combined solid-phase strategy was used to carry out the synthesis of β²-peptide **3** on MBHA resin (0.05 mmol). N^α-Alloc-N^β-Fmoc-L-diaminopropionic acid (2 equiv) was coupled to the resin in the presence of BOP (1.95 equiv), HOBT (2 equiv), and NMM (4.5 equiv) in DMF for 2.5 h at room temperature. Following deallylation (35 min x 3) of the side chain Alloc, coupling of side chain amino group was carried out using RCOOH (2 equiv), BOP (1.95 equiv), HOBT (2 equiv), and NMM (4.5 equiv) in DMF for 3 h at 25 °C. After washing and removal of the Fmoc

group, the reaction sequence was repeated to obtain **3**. Both the backbone elongation and the side chain coupling were monitored by the Kaiser test as well as test cleavage. After cleavage from the resin, the crude peptide was reconstituted in 30% CH₃CN and purified on a semipreparative Vydac C18 HPLC column (10 x 250 mm, flow rate = 2 mL/min, monitored at 220 nm) using a gradient of 10-35 % CH₃CN in 0.05% aqueous TFA over a period of 1 h. The identity and purity of the β²-hexapeptide **3** were assessed by analytical HPLC and MALDI-TOF mass spectrometry. ¹H NMR [CF₃CD₂OH, 500 MHz, 10 °C]: δ 0.96 (isovaleric methyls), 1.16 (isopropyl methyls), 1.72 (side chain methylenes, CH₂CH₂CH₂CH₂NH₃), 2.06 (isovaleric CH), 2.16 (isovaleric CH₂), 2.38 (side chain methylenes, CH₂CH₂CH₂CH₂NH₃), 2.51 (isopropyl CH), 3.02 (side chain methylenes, CH₂CH₂CH₂CH₂NH₃), 3.27-3.82 (backbone CH₂), 4.40-4.72 (backbone CH), 7.31 (side chain amine), 7.28, 7.33, 7.38 (phenyl ring), 7.36-8.16 (backbone and side chain amides). Due to broad lines resulting from aggregation at concentrations necessary for NMR analysis, sequential assignments could not be obtained. MALDI-TOF for C₄₉H₈₂N₁₄O₁₂, [M+H]⁺ calcd. 1059.62, found 1059.54 and [M+Na]⁺ calcd. 1081.62, found 1081.51; overall yield 42%.

Mixed α/β³-peptide 4. Solid-phase synthesis was used to carry out the synthesis of mixed α/β-peptide **4** on Wang resin. Fmoc/allyl combined solid-phase strategy was used to add β³-amino acids to the resin, where as, Fmoc-α-amino acids were coupled to the resin in single step. N-α-Fmoc-L-aspartic acid α-allyl ester (2 equiv) was coupled to Wang resin (0.1 mmol) in the presence of BOP (1.95 equiv), HOBT (2 equiv), and NMM (4.5 equiv) in DMF for 2 h at room temperature. After coupling, the resin was washed with DMF and DCM. Coupling was monitored with the Kaiser test. Following deprotection of the side chain All, coupling of the carboxyl was carried out using corresponding amine (RNH₂, 5 equiv) and the same coupling agents as above for 6-8 h at 25 °C. The resin was washed with DMF and DCM followed by removal of the N^α-Fmoc group and the sequence of reactions was repeated to couple β³-amino acids. Fmoc-α-amino acids were coupled using the same coupling

reagents as mentioned above to complete the synthesis. Mixed α/β -peptide **4** was cleaved from the resin using cleavage reagent (5 mL, 95:2:3, TFA/H₂O/triisopropylsilane) at room temperature for 2 hours and then washing the resin with the cleavage reagent (2 x 2 mins, 3 mL). The cleaved peptide was collected, combined with TFA washes, and concentrated by rotary evaporation. Cold diethyl ether (~ 10 mL) was added to precipitate the crude cleaved peptide. After trituration for 2 mins, the peptide was collected upon centrifugation and decantation of the ether. The crude peptide was reconstituted in 30% CH₃CN and purified on a semipreparative Vydac C18 HPLC column (10 x 250 mm, flow rate = 2 mL/min, monitored at 220 nm) using a gradient of 10-40% CH₃CN in 0.05% aqueous TFA over a period of 50 min. The identity and purity of α/β -undecamer peptide **4** were assessed by analytical HPLC and MALDI-TOF mass spectrometry. ¹H NMR [CF₃CD₂OH, 500 MHz, 10°C]: δ 0.93 (isovaleric methyls), 1.16 (isopropyl methyls), 1.59 (side chain methylenes, CH₂CH₂CH₂CH₂NH₃), 1.80 (isovaleric CH), 1.80 (side chain methylenes, CH₂CH₂CH₂CH₂NH₃), 2.03 (side chain methylenes, CH₂CH₂CH₂CH₂NH₃), 2.15-2.27 (glutamic acid CH₂CH₂COOH), 2.56 (glutamic acid CH₂CH₂COOH), 3.07 (isovaleric CH₂), 3.08 (side chain methylenes, CH₂CH₂CH₂CH₂NH₃), 2.80-2.90 (backbone CH₂), 4.01 (isopropyl CH), 4.10 (lysine CH), 4.36 (glutamic acid CH), 4.74 (backbone CH), 7.0-7.6 (side chain amides and lysine amine), 8.1-8.7 (backbone amides). Due to broad lines resulting from aggregation at concentrations necessary for NMR analysis sequential assignments could not be obtained. MALDI-TOF for C₇₄H₁₃₀N₂₀O₂₃, [M+H]⁺ calcd. 1667.00, found 1667.37 and [M+Na]⁺ calcd. 1689.00, found 1689.36; overall yield 64%.

α -peptide 5 . α -hexapeptide **5** (H-Val-Lys-Leu-Val-Lys-Leu-NH₂) was synthesized on MBHA resin using standard Fmoc-solid phase synthesis. The crude peptide was purified using semipreparative Vydac C18 HPLC column (10 x 250 mm, flow rate = 2 mL/min, monitored at 220 nm) using a gradient of 10-40 % CH₃CN in 0.05% aqueous TFA over a period of 50 min. The identity and purity of α -peptide **5** were assessed by analytical HPLC and MALDI-TOF mass spectrometry. MALDI-TOF for

$C_{34}H_{67}N_9O_6$, $[M+H]^+$ calcd. 698, found 698.2 and $[M+Na]^+$ calcd. 720.02, found 720.20; overall yield 83%.

Enzymatic Stability

Enzymatic degradation of peptides **1-5** using three enzymes, namely, pronase (*Streptomyces griseus*), trypsin (porcine pancreas), and elastase (hog pancreas) was carried out by incubation of peptides with the enzyme at 37 °C.

Proteolysis degradation buffers. The following reaction buffers were used to assay the select peptides in this study: 10 mM PBS at pH 7.5 for trypsin and elastase; 10 mM PBS at pH 7.8 for pronase. Peptides **1-5** employed in the degradation study were prepared as stock solution of 1 mM in PBS (10 mM, pH 7.5). Peptides were diluted with the select buffer before incubation with the enzyme.

Proteolytic stability assay. Enzymatic degradation was carried out by incubation of peptide (900 μ L, 350 μ M) with the enzyme (150 μ L, 60 μ M) at 37 °C for five days. The enzyme concentrations of the stock solutions were selected such that the α -hexapeptide **5** was totally degraded after a maximum of 1 h. The enzyme concentrations used completely degraded **5** by pronase in 15 minutes, trypsin in 30 minutes, and elastase in 1 h. Aliquots (70 μ L) were periodically taken at 0 h to 5 days, 5 μ L of 25% AcOH (*v/v*) were added, and the degradation was monitored by RP-HPLC.

Serum stability studies. Enzymatic degradation using human serum (Gemini Bio-Products, USA) was carried out by incubating peptides at 37 °C with the serum at peptide-serum ratio 8:1 in PBS, using 100 μ M final peptide concentration. Aliquots (95 μ L) were periodically taken at 0 h, 5 h, and 24 h, poured into 100 μ L of methanol to precipitate the proteins, and cooled on ice for 30 minutes. The sample was centrifuged and the supernatant was analyzed by RP-HPLC.

Cytotoxicity Assay

Cell culture. HeLa and COS-1 cells were maintained in RPMI-1640 (with L-glutamine and NaHCO₃) culture medium (Sigma) containing 10% fetal calf serum (FCS), 50 µg/mL penicillin, and 0.05 g/mL streptomycin in a humidified atmosphere (5% CO₂). Growing cells were detached from the culture flasks using a trypsin 0.25% EDTA solution, and the cell suspension was seeded on Corning 96-well plates (Corning Inc., NY, USA). Cells were incubated with the peptides in RPMI-1640 medium at 37 °C in CO₂ atmosphere.

MTT assay. Cytotoxicities of β-peptides **1** and **2** were determined by measuring the inhibition of cell growth using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (19). Cells were seeded in 96-well plates at a concentration of 1.0×10^4 cells/well/100 µL in complete media and incubated at 37 °C in 5% CO₂ atmosphere. After 24 h, the culture medium was discarded and replaced with a new medium containing peptides at a final concentration of 1, 10, 20, and 40 µM for incubation with HeLa and COS-1 cell lines. All the assays were performed in two independent sets of quadruplicate including positive control (TAT peptide) and negative control (media only) groups.

After exposure of the cells to the peptides for 24 h, the culture medium was discarded, and MTT (0.5 mg/mL) was added to each well. Cells were incubated with MTT for 3 h. Following incubation, the medium was removed and the purple formazan product precipitated in each well was solubilized in DMSO (200 µL). After gentle mixing for 5 min at room temperature, absorbance was measured at 570 nm using a VERSA max microplate reader (Molecular Devices) with a reference wavelength of 650 nm to subtract background. The percentage cell viability was expressed as the absorbance ratio of cells treated with peptides to untreated cells. Untreated cell were used as a negative control and wells with culture media but without the cells served as a blank control.

Results

Peptides **1-4** with different backbones, namely, β^3 , β^2 , or mixed α/β^3 , and varying side chains mimicking hydrophobic (valine and isoleucine), aromatic (phenylalanine), and charged (lysine and aspartic acid) amino acids were chosen for evaluation of biological stability and cellular toxicity. The sequence (or side chains) of β^3 -peptide **1** and β^2 -peptides **2** and **3** was kept very similar to the reported “regular” β -peptides (β -peptides synthesized from homologated α -amino acids) (1) to allow comparison of their biological stabilities. Mixed α/β^3 -undecamer peptide **4** was prepared to study the influence of a substituted α -amino acid in the β -peptide backbone. An α -hexapeptide **5** with the same side chains as β -peptides **1** and **2** was synthesized as a standard (or positive control) for enzymatic stability studies. β -peptides **1** and **2** were synthesized and characterized previously (15), while the synthesis of β^2 -peptide **3** and mixed α/β -peptide **4** is reported. β^3 - and β^2 -peptides were prepared utilizing a previously described Fmoc/allyl combined solid-phase strategy (15), using orthogonally protected L-Asp and L-Dap monomers, respectively. The β^3 -backbone is prepared from N $^\alpha$ -Fmoc-L-aspartic acid α -allyl ester, the β^2 -backbone from N $^\alpha$ -Alloc-N $^\beta$ -Fmoc-L-diaminopropionic acid, and the α -peptide backbone from N $^\alpha$ -Fmoc-L-amino acid monomers. The backbone is built by coupling the corresponding monomers using BOP and HOBt as coupling agents. The side chains for the β^3 - and β^2 -backbones are introduced by removal of the allyl group, followed by coupling of a protected amine or an acid, respectively, using the same coupling reagents as mentioned above.

To monitor the proteolytic degradation, peptides **1-4** were incubated with pronase, trypsin or elastase, and the stability of the peptides was monitored using reversed-phase HPLC analysis. This procedure allowed isolation of the possible degradation products which could then be characterized by electrospray ionization mass spectrometry (ESI-MS). All the enzymes were used in high specific

concentrations allowing complete degradation of α -peptide **5** within an hour. As shown in Table 1 and Fig. 2, β^3 -peptide **1** and β^2 -peptides **2** and **3** showed no sign of any degradation up to 4 days. After incubation with pronase, trypsin, or elastase, the HPLC peaks were collected and characterized by mass spectrometry. The collected peaks showed the same mass as the original peptides, suggesting that no cleavage occurred. β -peptides **1** and **2** also did not display any degradation in the presence of human serum when incubated with the serum for 5 days, while α -peptide **5** was completely degraded within 24 h under similar conditions.

Table 1. Treatment of peptides **1-5** with three proteolytic enzymes or human serum.

Fig. 2 - RP-HPLC chromatograms of β -peptides **1**, **2**, and **3** in the presence of pronase at different time intervals (0 hour and 4 days) illustrating the enzymatic stability.

Interestingly, the insertion of a single α -amino acid between four β -amino acid units rendered mixed α/β^3 -undecamer peptide **4** susceptible to degradation. Mixed α/β -peptide **4** displayed variable degradation in the presence of pronase and trypsin, while retaining complete stability in the presence of elastase. As shown in Table 2, proteolytic degradation of mixed α/β^3 -peptide **4** started within an hour and was fully degraded by pronase and trypsin in 24 h. After incubation with pronase for 2 h, the two additional peaks that eluted at 12 and 13 minutes, along with **4** eluting at 16 minutes, became more prominent (Fig. 3). The mass $[M+H]^+$ of the peaks eluting at 12 and 13 minutes was found to be 630.23 and 1056.23, respectively. This corresponded to peptide bond cleavage between residues Lys7 and $\beta^3\text{amVal8}$ ($L\text{-}\beta^3\text{-amidovaline8}$) giving rise to heptapeptide **6** ($[M+H]^+_{\text{calcd.}} 1056.63$) and tetrapeptide **7** ($[M+H]^+_{\text{calcd.}} 630.33$). Further incubation with pronase led to complete loss of **4** with the appearance

of multiple additional peaks at around 8 and 14 minutes. Mass spectrometric analysis of the two peaks that eluted at ~ 14 minutes showed $[M+H]^+$ of 1056.45 and 928.36. This suggested that the N-terminal fragment **6** was further hydrolyzed after Lys1 to give a hexapeptide **8** ($[M+H]^+_{\text{calcd.}}$ 928.53). In the presence of trypsin, mixed α/β -peptide **4** degraded to mainly two peaks eluting at 12 and 13 minutes. The mass of these peaks was found to be 629.80 and 1056.06, respectively, suggesting cleavage after Lys7. After 4 days of incubation, the parent peak for **4** was not observed, while peaks at 12 and 13 minutes corresponding to fragments **7** and **6**, respectively, were still present as shown in Fig. 3.

Table 2. Degradation of peptide **4** in the presence of three proteolytic enzymes.

Fig. 3 - RP-HPLC chromatograms of peptide **4** after incubation with pronase (top) and trypsin (bottom) for different time intervals, namely, 0 hr (red), 2 hr (blue), and 4 days (black). On the left side are shown the molecular formulae of the final degradation product observed by mass spectrometry in the presence of pronase and trypsin.

In addition, to obtain a general view of the toxic profile displayed by the β -peptides discussed here, the cytotoxic properties of representative β -peptides **1** and **2** were assessed in HeLa and COS-1 cells. The toxicities were determined by MTT assay (19) and estimated to be lower than that of TAT peptide at the same concentration. Cell penetrating peptide, TAT₄₇₋₅₇, was used as a positive control for cell cytotoxicity assays because of the cytotoxic effects of TAT on several cell lines (20, 21). The viability of HeLa and COS-1 cells after treatment with β^2 -peptide **2** for 24 hours was always higher than 95% at all the concentrations used (1-40 μM) as shown in Fig. 4. β^3 -peptide **1** displayed slight cytotoxicity in HeLa and COS-1 cells at the highest concentration used (40 μM), with a viability of $88 \pm 2\%$ and $92 \pm$

1%, respectively. At this concentration (40 μ M), TAT peptide exhibited significant toxicity (71-76%) in both the cell lines.

Fig. 4 - Cytotoxicity of β -peptides **1** (white) and **2** (grey) compared to TAT (black) monitored in (A) HeLa and (B) COS-1 cell lines using MTT assay. Cells were incubated with different concentration of peptides for 24 hours. □

Discussion

The proteolytic susceptibility of the peptides discussed in this report was evaluated against three widely used proteases, namely, pronase, trypsin, and elastase from different biological sources. Trypsin and elastase are peptidases that cleave peptide bonds at specific residues (22, 23). Pronase, however, is a mixture of several exo and endopeptidases and was selected due to its high activity and broad substrate specificity (24, 25). The “pure” β -peptides **1-3** derived from L-Asp and L-Dap were resistant to cleavage by all the three enzymes (Table 1). In spite of possessing amide groups on the α -carboxylate (as shown in Fig. 5) and α -amine of the aspartate and diaminopropionate residues, respectively, the side chain amides of β -peptides **1-3** were not cleaved on treatment with the three enzymes, behavior consistent with the “regular” β -peptides.

Fig. 5 - β^3 -peptide prepared from L-aspartic acid monomers contain substituted L- α -amino acid (substituted L-asparagine) residues (highlighted in the dashed box).

Mixed α/β -peptide **4** was cleaved by two of the proteases (pronase and trypsin) at the amidic bonds followed by Lys residues as depicted in Fig. 6. Proteolysis of □ α/β -peptides has been observed

previously, however, mainly by pronase (2, 5). After incubation with pronase, **4** lost its N-terminal Lys residue (as expected) and was also cleaved between residues 7 (Lys) and 8 (a β -Asp derived residue). This was an unexpected result because cleavage of a central α/β -peptide bond by proteases has been considered difficult (2, 5). Interestingly, the later bond between residues 7 and 8 was also cleaved by trypsin. Trypsin, a serine endopeptidase that cleaves peptide bonds adjacent to the basic residues (such as Arg or Lys), cleaved **4** after Lys7. This suggests that the Lys7/ β^3 -amVal8 bond may be susceptible to cleavage by pronase and trypsin as it represents an α/α -peptide bond with the correct absolute configuration. However, the rate of hydrolysis of this bond was significantly lower than that would be expected for an α/α -peptide bond in an α -peptide. Complete proteolysis of mixed α/β -peptide **4** occurred in 24 h (Table 2), whereas, α -peptide **5** was degraded by pronase in 15 minutes and by trypsin in 30 minutes under similar conditions. These results suggest that the use of L-Asp monomers to insert a β -peptide backbone in a biologically active peptide may have an additional advantage.

Fig. 6 - Degradation of mixed α/β^3 -peptide by proteases. α - and β -amino acids are shown in grey and black, respectively.

In summary, the results presented here show that the insertion of α -amino acid residues in a β -peptide derived from L-Asp monomers increases substrate-target recognition, retains the proteolytic degradation resistance, and imparts no cytotoxicity.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC). The authors would like to thank Dr. Tara Sprules from Quebec/Eastern Canada High Field NMR centre for performing NMR experiments.

References

1. Frackenpohl J, Arvidsson PI, Schreiber JV, Seebach D. (2001) The outstanding biological stability of beta- and gamma-peptides toward proteolytic enzymes: an in vitro investigation with fifteen peptidases. *Chembiochem*;2:445-55.
2. Hook DF, Bindschadler P, Mahajan YR, Sebesta R, Kast P, Seebach D. (2005) The proteolytic stability of 'designed' beta-peptides containing alpha-peptide-bond mimics and of mixed alpha,beta-peptides: application to the construction of MHC-binding peptides. *Chem Biodivers*;2:591-632.
3. Horne WS, Gellman SH. (2008) Foldamers with heterogeneous backbones. *Acc Chem Res*;41:1399-408.
4. Porter EA, Weisblum B, Gellman SH. (2002) Mimicry of host-defense peptides by unnatural oligomers: antimicrobial beta-peptides. *J Am Chem Soc*;124:7324-30.
5. Schmitt MA, Weisblum B, Gellman SH. (2007) Interplay among folding, sequence, and lipophilicity in the antibacterial and hemolytic activities of alpha/beta-peptides. *J Am Chem Soc*;129:417-28.
6. Seebach D, Gardiner J. (2008) Beta-peptidic peptidomimetics. *Acc Chem Res*;41:1366-75.
7. Hamuro Y, Schneider JP, DeGrado WF. (1999) De Novo Design of Antibacterial beta-Peptides. *J Am Chem Soc*;121:12200-1.

8. LePlae PR, Fisk JD, Porter EA, Weisblum B, Gellman SH. (2002) Tolerance of acyclic residues in the beta-peptide 12-helix: access to diverse side-chain arrays for biological applications. *J Am Chem Soc*;124:6820-1.
9. English EP, Chumanov RS, Gellman SH, Compton T. (2006) Rational development of beta-peptide inhibitors of human cytomegalovirus entry. *J Biol Chem*;281:2661-7.
10. Gademann K, Kimmerlin T, Hoyer D, Seebach D. (2001) Peptide folding induces high and selective affinity of a linear and small beta-peptide to the human somatostatin receptor 4. *J Med Chem*;44:2460-8.
11. Kritzer JA, Lear JD, Hodsdon ME, Schepartz A. (2004) Helical beta-peptide inhibitors of the p53-hDM2 interaction. *J Am Chem Soc*;126:9468-9.
12. Hetenyi A, Mandity IM, Martinek TA, Toth GK, Fulop F. (2005) Chain-length-dependent helical motifs and self-association of beta-peptides with constrained side chains. *J Am Chem Soc*;127:547-53.
13. Aguilar MI, Purcell AW, Devi R, Lew R, Rossjohn J, Smith AI, et al. (2007) Beta-amino acid-containing hybrid peptides--new opportunities in peptidomimetics. *Org Biomol Chem*;5:2884-90.
14. Hook DF, Gessier F, Noti C, Kast P, Seebach D. (2004) Probing the proteolytic stability of beta-peptides containing alpha-fluoro- and alpha-hydroxy-beta-amino acids. *Chembiochem*;5:691-706.
15. Ahmed S, Beleid R, Sprules T, Kaur K. (2007) Solid-phase synthesis and CD spectroscopic investigations of novel beta-peptides from L-aspartic acid and beta-amino-L-alanine. *Org Lett*;9:25-8.
16. Kaur K, Sprules T, Soliman W, Beleid R, Ahmed S. (2008) Right-handed 14-helix in beta 3-peptides from L-aspartic acid monomers. *Biochim Biophys Acta*;1784:658-65.
17. Heck T, Limbach M, Geueke B, Zacharias M, Gardiner J, Kohler HP, et al. (2006) Enzymatic degradation of beta- and mixed alpha,beta-oligopeptides. *Chem Biodivers*;3:1325-48.

18. Hiraishi T, Kajiyama M, Yamato I, Doi Y. (2004) Enzymatic hydrolysis of alpha- and beta-oligo(L-aspartic acid)s by poly(aspartic acid) hydrolases-1 and 2 from *Sphingomonas* sp. KT-1. *Macromol Biosci*;4:330-9.
19. Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*;65:55-63.
20. El-Andaloussi S, Jarver P, Johansson HJ, Langel U. (2007) Cargo-dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: a comparative study. *Biochem J*;407:285-92.
21. Farrera-Sinfreu J, Giralt E, Castel S, Albericio F, Royo M. (2005) Cell-penetrating cis-gamma-amino-l-proline-derived peptides. *J Am Chem Soc*;127:9459-68.
22. Corey DR, Willett WS, Coombs GS, Craik CS. (1995) Trypsin specificity increased through substrate-assisted catalysis. *Biochemistry*;34:11521-7.
23. Largman C, Brodrick JW, Geokas MC. (1976) Purification and characterization of two human pancreatic elastases. *Biochemistry*;15:2491-500.
24. Narahashi Y, Shibuya K, Yanagita M. (1968) Studies on proteolytic enzymes (pronase) of *Streptomyces griseus* K-1. II. Separation of exo- and endopeptidases of pronase. *J Biochem*;64:427-37.
25. Narahashi Y, Yanagita M. (1967) Studies on proteolytic enzymes (pronase) of *Streptomyces griseus* K-1. I. Nature and properties of the proteolytic enzyme system. *J Biochem*;62:633-41.

TABLES

Table 1. Treatment of peptides **1-5** with three proteolytic enzymes or human serum.

Enzyme or Serum	Enzyme origin	1 (β^3)	2 (β^2)	3 (β^2)	4 (α/β^3)	5 (α)
Pronase	<i>Streptomyces griseus</i>	-	-	-	+	+
Trypsin	porcine pancreas	-	-	-	+	+
Elastase	hog pancreas	-	-	-	-	+
Human serum		-	-	n.t.	n.t.	+

(+) sign indicates degradation, whereas (-) sign indicates no detectable degradation under the experimental conditions. n.t.: not tested

Table 2. Degradation of peptide **4** in the presence of three proteolytic enzymes.

Time (h)	% Intact Peptide ^[a]		
	Pronase	Trypsin	Elastase
0	100	100	100
0.5	100	100	100
1	76	88	100
2	51	75	100
5	21	50	100
24	0	0	100
4 days	0	0	100

[a] % intact peptide was calculated based on the area under the HPLC peak for the parent peptide.

FIGURES

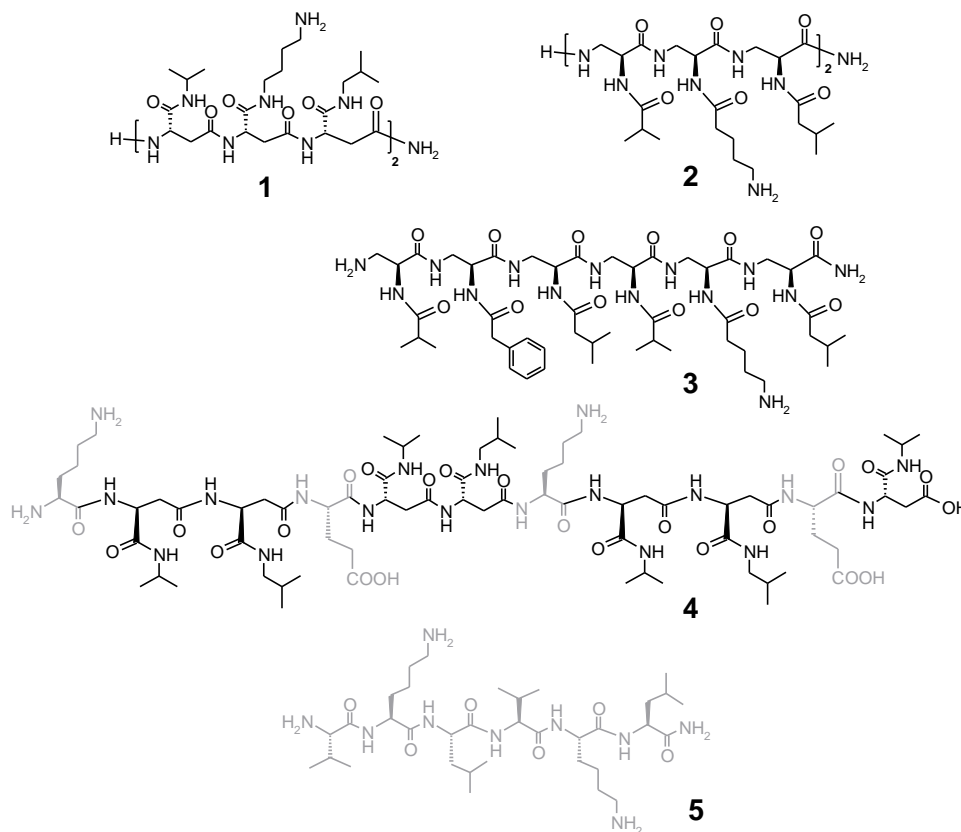


Fig. 1 - Chemical structures of peptides **1-5** studied herein. α -amino acids are shown in grey.

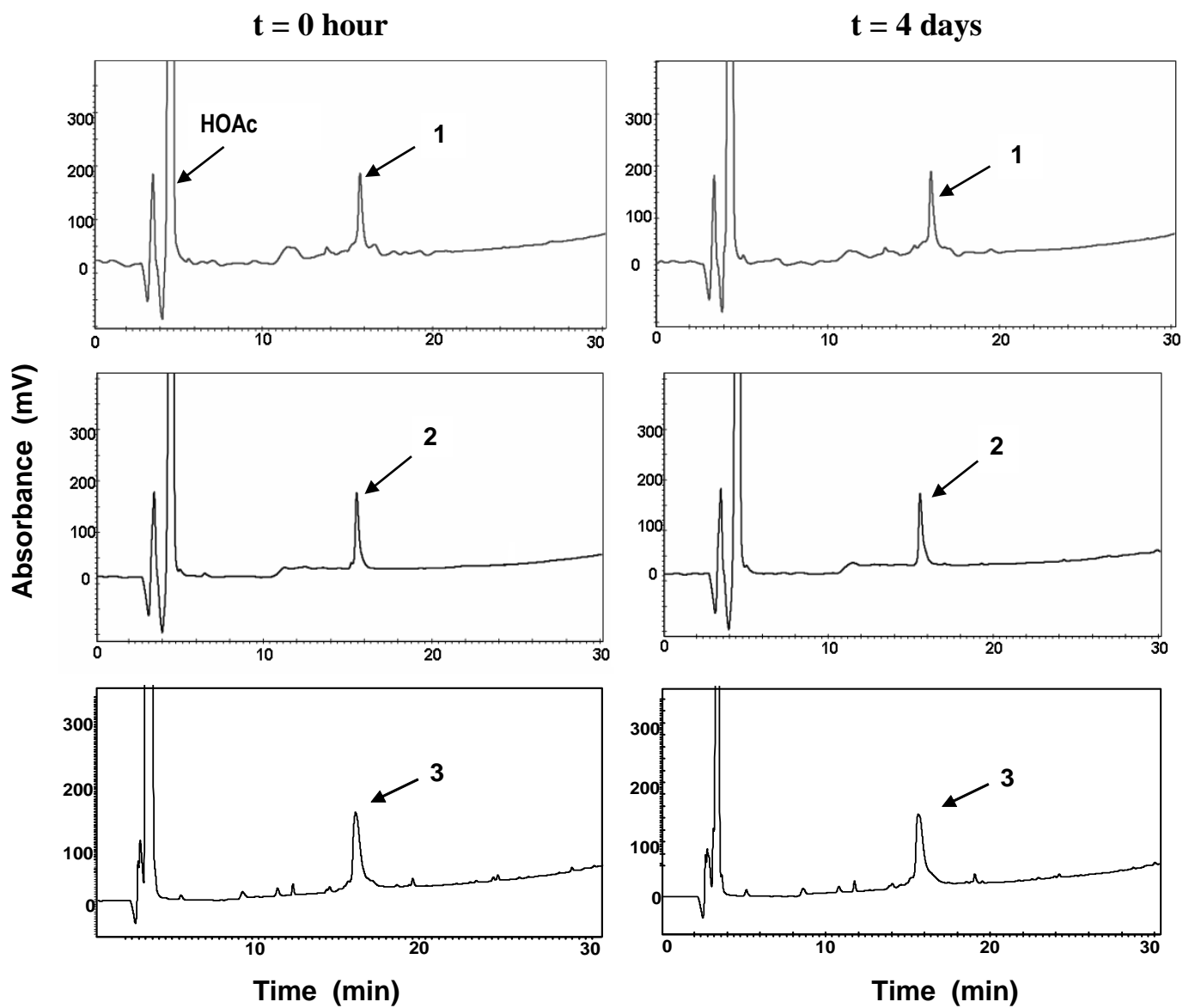


Fig. 2 - RP-HPLC chromatograms of β -peptides **1**, **2**, and **3** in the presence of pronase at different time intervals (0 hour and 4 days) illustrating the enzymatic stability.

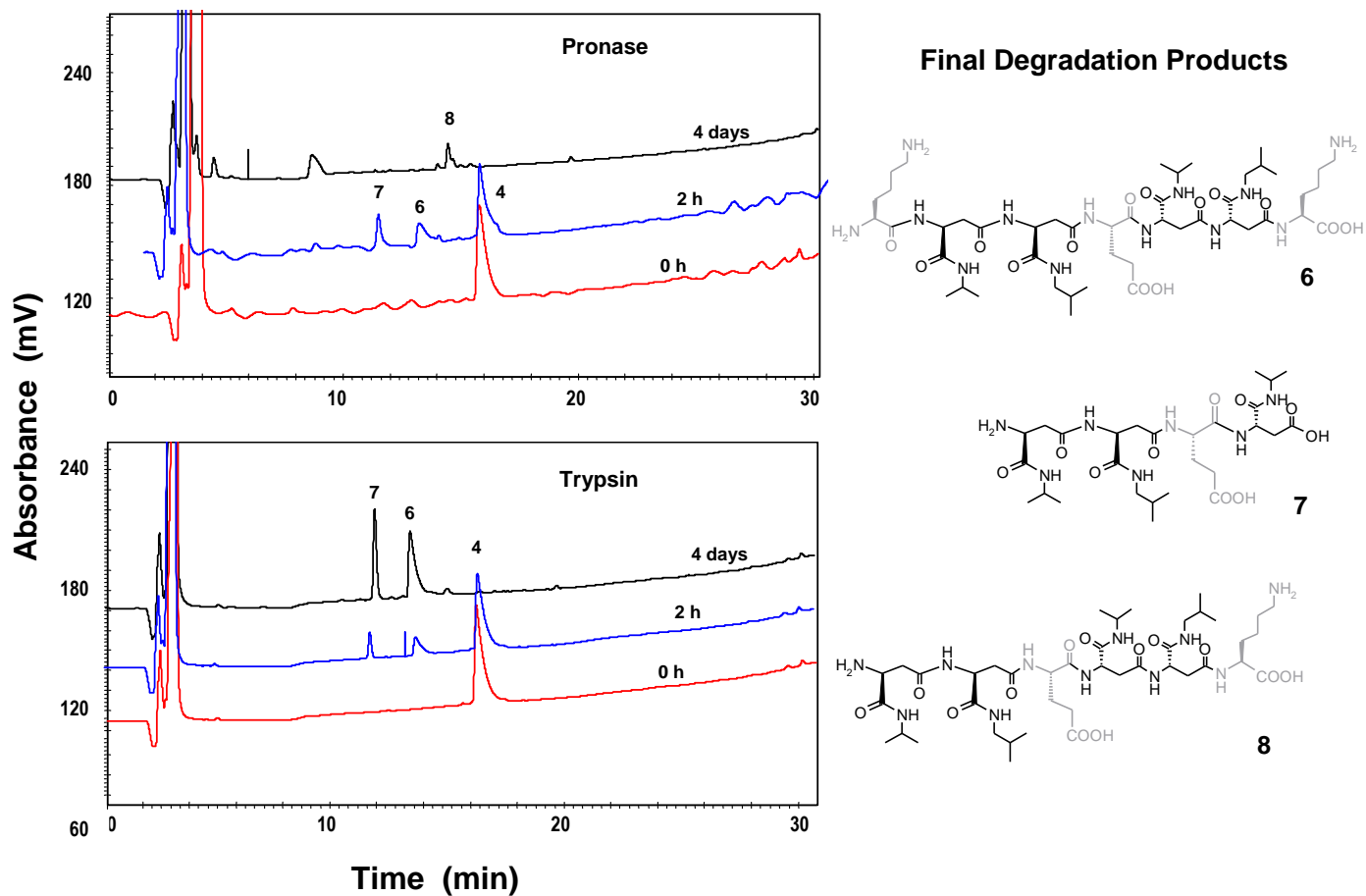


Fig. 3 - RP-HPLC chromatograms of peptide **4** after incubation with pronase (top) and trypsin (bottom) for different time intervals, namely, 0 hr (red), 2 hr (blue), and 4 days (black). On the left side are shown the molecular formulae of the final degradation product observed by mass spectrometry in the presence of pronase and trypsin.

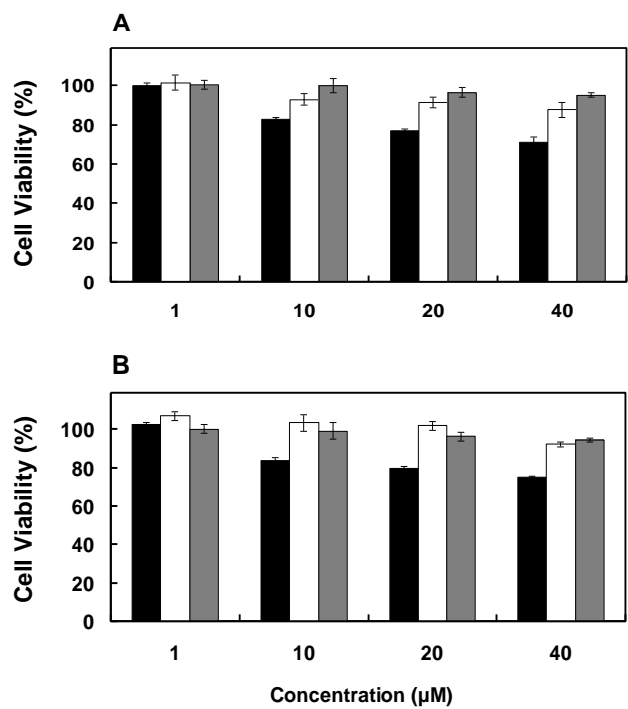


Fig. 4 - Cytotoxicity of β -peptides **1** (white) and **2** (grey) compared to TAT (black) monitored in (A) HeLa and (B) COS-1 cell lines using MTT assay. Cells were incubated with different concentration of peptides for 24 hours. □

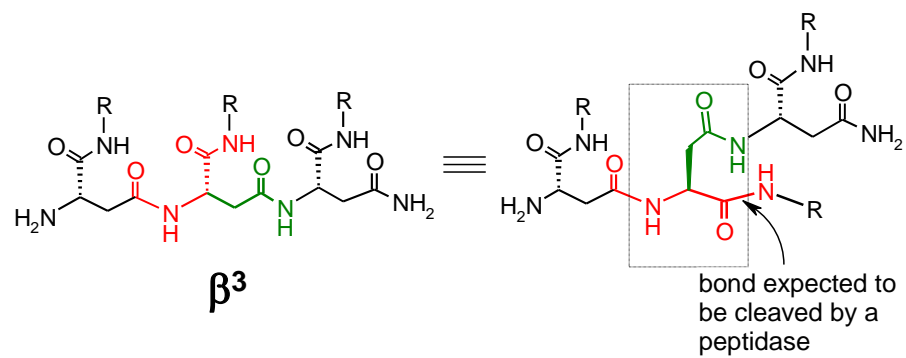


Fig. 5 - β^3 -peptide prepared from L-aspartic acid monomers contain substituted L- α -amino acid (substituted L-asparagine) residues (highlighted in the dashed box).

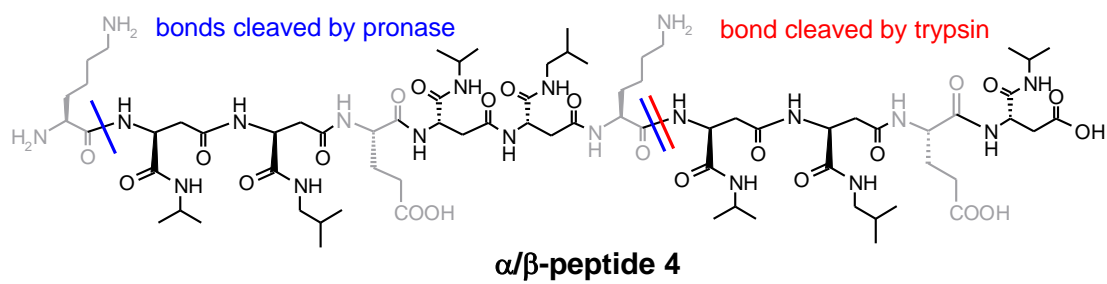


Fig. 6 - Degradation of mixed α/β^3 -peptide 4 by proteases. α - and β -amino acids are shown in grey and black, respectively.