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Mass spectrometric identification of the trypsin cleavage pathway in lysyl-proline containing oligotuftsin peptides

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Abstract: Trypsin cleaves specifically peptide bonds at the *C*-terminal side of lysine and arginine residues, except for -Arg-Pro- and -Lys-Pro- bonds which are normally resistant to proteolysis. Here we report evidence for a -Lys-Pro- tryptic cleavage in modified oligotuftsin derivatives, Ac-$[TKPKG]_4$-NH_2) (**1**), using high-resolution mass spectrometry and HPLC as primary methods for analysis of proteolytic reactions. The proteolytic susceptibility of -Lys-Pro- bonds was strongly dependent on flanking residues, and the flexibility of the peptide backbone might be a prerequisite for this unusual cleavage. While -Lys-Gly- bonds in **1** were rapidly cleaved, the modification of these Lys residues by the attachment of a ß-amyloid(4–10) epitope to yield -Lys(X)-Gly derivatives prevented cleavage of this bond, and provided trypsin cleavage of -Lys-Pro- bonds, the pathway of this degradation being independent on the type of Lys-N^{ε}-side chains (acetyl group, amino acid, peptide). Substitution of the Lys residues by Ala at the P′2 positions decreased the tryptic cleavage, while replacement of the bulky side chain of Thr at the P2 positions strongly increased the cleavage of -Lys-Pro- bonds. Circular dichroism (CD) data of the modified oligotuftsin derivatives are in accord with enhanced flexibility of the peptide backbone, as a prerequisite for increased susceptibility to cleavage of -Lys-Pro- bonds. These results obtained of oligotuftsin derivatives might have implications for the proteolytic degradation of target peptides that require specific conformational preconditions. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: trypsin specificity; -Lys-Pro- cleavage; oligotuftsin derivatives; high-resolution FTICR mass spectrometry

INTRODUCTION

Trypsin is one of the most specific proteolytic enzymes and is most frequently used in protein structure analysis. Trypsin belongs to the serine endopeptidase family S1, which cleaves peptide bonds specifically at the *C*-terminal side of lysine and arginine residues. The substrate-binding pocket of trypsin has a negative charge derived from the Asp-189 residue (in bovine trypsin) which is responsible for the specific binding of the enzyme to positively charged amino acid side chains of the substrate *via* ionic interaction [1,2]. The rate of hydrolysis is substantially affected by the nature of the bond (amide or imide) between amino acid residues in positions P1 and P′1 (Figure 1), and by the structure of flanking sequences. Peptide bonds at the *C*-terminal of Arg are generally cleaved faster (2- to10-fold) than at Lys residues [3–5]. The presence of acidic residues in positions P2 or P′2 of the cleavage site may lead to considerably reduced cleavage rates or even total resistance to cleavage [4,5]. The presence of a positive charge, particularly adjacent Lys and Arg residues or the vicinity to the free *N*-terminal amino group, may also reduce the rate of cleavage [4].

Despite the high specificity of trypsin, nonspecific cleavage at the *C*-terminal of other amino acid residues has been observed in several cases at prolonged reaction times. In such cases, the appearance of pseudotrypsin (Ψ-trypsin) has been reported which possesses chymotrypsin-like specificity and hydrolyzes bonds adjacent to aromatic residues [6,7]. The chymotryptic activity may be inactivated by treatment with L-(1-tosylamido-2-phenyl) ethylchloromethyl ketone (TPCK) [8] or by methylation, which makes the enzyme more resistant to autolysis [9]. Recently, using high-resolution Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), such modified trypsin has been shown to cleave at the *C*-terminal of arginine and lysine residues with high specificity, without formation of nontryptic peptides [10]. Restrictions to the specificity of trypsin occur when proline is at the carboxylic side of lysine or arginine, such bonds being highly resistant to cleavage [3–5,11,12]. The ring structure in proline restricts the freedom of rotation, which renders the polypeptide backbone more rigid. Although proline in position P′1 usually blocks trypsin cleavage, the -Lys-Pro- bond can be cleaved when Lys in position P1 and Trp in position P2 are present simultaneously. Similarly, the presence of Arg in position P1 and Met in position P2 resulted in efficient trypsin hydrolysis. The inhibitory effect of Pro is also superseded by the

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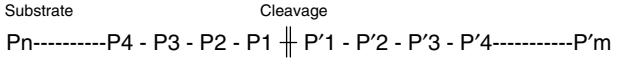

Figure 1 Schematic representation of a substrate. Positions Pn and P′m in the substrate are counted from the bond between P1 and P′1, where the cleavage occurs.

presence of Glu at position P2 [13]. The cleavage of -Arg-Pro- bonds has been also described in human acid sphingomyelinase [14], in the sequences -Trp-Arg-Pro-Ala-, -Ala-Arg-Pro-Ala- [4] and in RYP**RP**AS(p)VPPS(p)P (where S(p) is phosphoserine) [15]. Examples of trypsin sensitive -Lys-Pro- bonds have been also reported in proline-rich proteins from human saliva [16,17] and in a hydroxyproline-rich glycoprotein from *Zea mays* [18]. Kieliszewski *et al.* [18] suggested two prerequisites for -Lys-Pro- cleavage: (i) the presence of an extended helix in proline- or hydroxyproline-rich regions of proteins and (ii) the flexibility of peptide bonds associated with two amino acid residues flanking the cleavage site. However, such proposals have not yet been experimentally ascertained.

In the present study, we report on the identification of -Lys-Pro- cleavage pathways by trypsin in branched polypeptide conjugates containing a ß-amyloid(4–10) (Aß(4–10)) plaque-specific epitope peptide (FRHDSGY) [19,20] attached to the Lys-Pro-containing peptide backbone of oligotuftsin [21], Ac-[TKPKG]$_4$-NH$_2$) (**1**). Analysis of the tryptic digestion of oligotuftsin derivative (**1**) and its conjugate with Aß(4–10) epitope peptide **2** (Ac-[TKPK(H-FRHDSGY)G]$_4$-NH$_2$) by HPLC and mass spectrometry revealed significant differences between their proteolytic degradation pathways. The oligotuftsin carrier (**1**) was rapidly digested by trypsin at the -Lys-Gly- cleavage site. The attachment of the Aß-epitope peptide to the side chains of the Lys-Gly residues (Lys-4, Lys-9, Lys-14 and Lys-19 in **1**) prevented cleavage at these sites, but provided tryptic fragments by cleavage at the Lys residues *N*-terminal to Pro residues, as identified by HPLC and FTICR-MS. In order to evaluate the potential effects of the type and length of the side-chain peptide attached to the lysine residues in tryptic digestion, oligotuftsin derivatives were studied in which single alanine branching residues were attached to the Lys-Gly sites (Ac-[TKPK(A)G]$_4$-NH$_2$ (**3**), and in which the ε-amino groups of the Lys-Gly residues were modified by acetylation (Ac-[TKPK(Ac)G]$_4$-NH$_2$ (**4**). Both oligotuftsin derivatives **3** and **4** showed degradation of Lys-Pro residues similar to the Aß(4–10)-modified conjugate **2**, as did an oligotuftsin derivative in which the Lys-Gly residues were replaced by alanine (Ac-[TKPAG]$_4$-NH$_2$; **5**). Furthermore, the additional substitution of threonine residues by alanine (Thr1/6/11/16Ala; Ac-[AKPAG]$_4$-NH$_2$ (**6**)) provided a peptide that showed rapid tryptic digestion at Lys-Pro residues. Comparative analyses of the oligotuftsin derivatives by CD spectroscopy indicated preferential random coil conformations both in the aqueous solution in which the tryptic digestion was carried out (10 mM NH_4HCO_3, pH 8) and in trifluoroethanol (TFE). However, oligotuftsin derivative **6** (Ac-[AKPAG]$_4$-NH$_2$) adopted a more ordered structure in TFE compared to the other investigated compounds, suggesting higher flexibility of the peptide backbone, which might be a key prerequisite for enhanced Lys-Pro cleavage by trypsin.

MATERIALS AND METHODS

Amino acid derivatives and MBHA resin were purchased from Reanal (Budapest, Hungary) and NovaBiochem (Läufelfingen, Switzerland). Scavengers, coupling agents and cleavage reagents were from Fluka (Buchs, Switzerland) (*p*-cresol, dithiothreitol (DTT), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-diisopropyl-ethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), piperidine, trifluoroacetic acid (TFA) and hydrogen fluoride). Acetic anhydride (Ac_2O) and solvents used for synthesis and purification were purchased from Reanal (Budapest, Hungary) and Riedel-deHäen (Seelze, Germany).

Peptide Synthesis

Peptides **1**, **5** and **6** (Ac-[TKPKG]$_4$-NH$_2$ (**1**), Ac-[TKPAG]$_4$-NH$_2$ (**5**), Ac-[AKPAG]$_4$-NH$_2$ (**6**)), were synthesized manually by SPPS according to the Boc/Bzl strategy using MBHA resin (1.04 mmol/g capacity) as a support. The following Boc-protected amino acid derivatives were used: Boc-Gly-OH, Boc-Lys(ClZ)-OH, Boc-Pro-OH, Boc-Thr(Bzl)-OH, Boc-Ala-OH. The following synthetic protocol was pursued: (i) deprotection with 33% TFA/DCM (2 + 20 min); (ii) DCM washing (5 × 0.5 min); (iii) neutralization with 10% DIEA/DCM (4 × 1 min); (iv) DCM washing (4 × 0.5 min); (v) coupling of 3 equivalents of Boc-amino acid derivative using DCC/HOBt in DCM-DMF 4 : 1 (60 min); (vi) DMF washing (2 × 0.5 min); (vii) DCM washing (2 × 0.5 min); (viii) ninhydrin or isatin assay to assess the presence of free amines. Acetylation of the *N*-terminus was carried out for 1 h with acetic anhydride (1 ml) and DIEA (1 ml) in DMF.

Peptides (**2**), (**3**) and (**4**) (Ac-[TKPK(H-FRHDSGY)G]$_4$-NH$_2$, Ac-[TKPK(A)G]$_4$-NH$_2$, and Ac-[TKPK(Ac)G]$_4$-NH$_2$)) were prepared according to the same protocol as described above. However, instead of using Boc-Lys(ClZ)-OH, Boc-Lys(Fmoc)-OH was utilized in positions 4, 9, 14 and 19 and Boc-Lys(ClZ)-OH in positions 2, 7, 12 and 17 of the backbone sequence. After completion of the synthesis of the oligotuftsin derivative, the N^{ε}-Fmoc protecting group of Lys was cleaved off with 2% DBU, 2% piperidine in DMF for 25 min; the free amino group was then either acetylated (peptide **4**), or Aß(4–10) peptide was built up on the ε-amino group of lysine using the Boc/Bzl strategy (conjugate **2**). The oligotuftsin derivative **3** was obtained by coupling of a single alanine residue to the carrier.

All peptides were cleaved from the resin with liquid HF in the presence of *p*-cresol and DTT as scavengers (HF-*p*-cresol-DTT, 10 ml: 0.5 g: 0.1 g) for 1.5 h at 0 °C. In the case of conjugate **2**, 0.1 g Cys was added to the cleavage mixture in order to remove formaldehyde derived from the Bom protecting group.

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The crude products were precipitated with diethyl ether and the filtrate was washed three times with diethyl ether and solubilized using 10% acetic acid before freeze-drying. The crude materials were purified by semipreparative RP-HPLC on a C_{18} column.

Trypsin Digestion

Peptides were dissolved in 10 mM NH_4HCO_3, pH 8, at a concentration of 1 μg/μl and incubated with sequence grade modified porcine trypsin (Promega; Madison, WI, USA; 1 μg/μl in 50 mM acetic acid) at an enzyme to substrate ratio of 1 : 50 (w/w) at 37 °C. Aliquots of 25 μl were taken at 60 min intervals, and the degradation products were directly analyzed by mass spectrometry or subjected to analytical HPLC separation on a C_{18} column. The HPLC fractions were collected and analyzed by MALDI-TOF- MS.

High-performance Liquid Chromatography

Analytical RP-HPLC was performed on a Bio-Rad 254 instrument (Bio-Rad Laboratories, Richmond, CA) using an analytical Nucleosil 300–7 C_{18} column (4 × 250 mm, 300 Å, 7 μm; Macherey-Nagel, Düren, Germany) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 55 min 100% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80 : 20 v/v)) was used at a flow rate of 1 ml/min at 20 °C. Peptides were detected at 220 nm.

Purification of linear and branched peptides was carried out on a Knauer preparative HPLC (Knauer, Berlin, Germany) using a 20 × 250 mm preparative C_{18} column (GROM-SIL 120 ODS-4 HE, 120 Å, 10 μm; Grom, Herrenberg-Kayh, Germany). Linear gradient elution was performed using the same mobile phases as described above (0 min 10% B; 5 min 10% B; 95 min 100% B), at a flow rate of 10 ml/min. The peptides were dissolved in eluent A, and peak detection was performed at 220 nm.

Mass Spectrometry

MALDI-FTICR mass spectrometric analysis was performed with a Bruker APEX II FTICR instrument equipped with an actively shielded 7 T superconducting magnet, a cylindrical infinity ICR analyzer cell, and an external Scout 100 fully automated *X–Y* target stage MALDI source with pulsed collision gas (Bruker Daltonics, Bremen, Germany). The pulsed nitrogen laser is operated at 337 nm. A 100 mg/ml solution of 2,5-dihydroxybenzoic acid (DHB, Aldrich, Steinheim, Germany) in acetonitrile: 0.1% TFA in water (2 : 1, v/v) was used as the matrix. Aliquots of 0.5 μl of the matrix solution and 0.5 μl of the sample solution were mixed on the stainless steel MALDI sample target and allowed to dry. Calibration was performed with a standard peptide mixture within an m/z range of approximately 4000.

MALDI-TOF-MS analysis was carried out with a Bruker Biflex linear TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen UV laser (337 nm), a dual channel plate detector, a 26-sample SCOUT source, a video system and an XMASS data system for spectra acquisition and instrument control. A saturated solution of α-cyano-4-hydroxy-cinnamic acid (HCCA) in acetonitrile/0.1% TFA in water (2 : 1, v/v) was used as the matrix. An aliquot of 1 μl of the sample solution was applied on the target and 1 μl of a freshly prepared saturated solution of HCCA in acetonitrile: 0.1% TFA in water (2 : 1, v/v) was added. Acquisition of MALDI spectra was carried out at an acceleration voltage of 20 kV and a detector voltage of 1.5 kV.

CD Spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco model J-715 spectropolarimeter at 20 °C in quartz cells of 0.05 cm path length, under a constant nitrogen flush. The instrument was calibrated with 0.06% (w/v) ammonium-*d*-camphor-10-sulfonate (Katayama Chemical, Japan) in water. TFE (Fluka, Buchs, Switzerland) and 10 mM NH_4HCO_3, pH 8, were used as solvents. The concentration of the samples was 200 μM. The spectra were averages of six scans in a wavelength range of 190–260 nm. Results are expressed in terms of mean residue ellipticity (deg cm^2 $dmol^{-1}$) after subtraction of the solvent baseline.

For the deconvolution of the CD spectra, the Dicroprot v2.4 software (Institute de Biologie et Chimie des Protéines, Lyon, France) was employed. The percentage of each secondary structure elements was calculated using the least-square fit with Fasman, Chen and Yang models. The content of α-helix was also calculated from the ellipticity.

Molecular Modeling

Molecular modeling calculations were performed by using the software Hyperchem 7.0. For geometry optimization, the Amber96 parameter set and the Polak–Ribiere algorithm with an RMS gradient of 0.001 kcal mol^{-1} $Å^{-1}$ were employed. The simulations were carried out at room temperature (298 K) in presence of water molecules. The size of the periodic box was dependent on the size of the investigated molecules (between 678 and 1363 water molecules were taken into account, the distance between solvent and solute atoms being 2.3 Å).

RESULTS AND DISCUSSION

In the present study we investigated an unusual -Lys-Pro- cleavage by trypsin, which occurs in modified oligotuftsin peptides (shown in Figure 2). The oligotuftsin peptide, Ac-$[TKPKG]_4$-NH_2 (**1**), contains eight lysine residues with either Pro or Gly at the *C*-terminal side: four -Lys-Pro- and four -Lys-Gly- bonds. As expected, the proteolytic digestion of peptide **1** with trypsin at 37 °C (at a 1 : 50 enzyme to substrate ratio) resulted in the rapid cleavage of the -Lys-Gly- bonds, while no cleavage of -Lys-Pro- bonds was detected. The MALDI-TOF mass spectrum (Figure 3(A)) and the HPLC profile of the tryptic mixture recorded after 15 min reaction (Figure 3(B)) showed the presence of only two peaks, corresponding to the fragments Ac-TKPK-OH (R_t = 15.8 min) and H-GTKPK-OH (R_t = 7.9 min) (Figure 3, Table 1). The composition of

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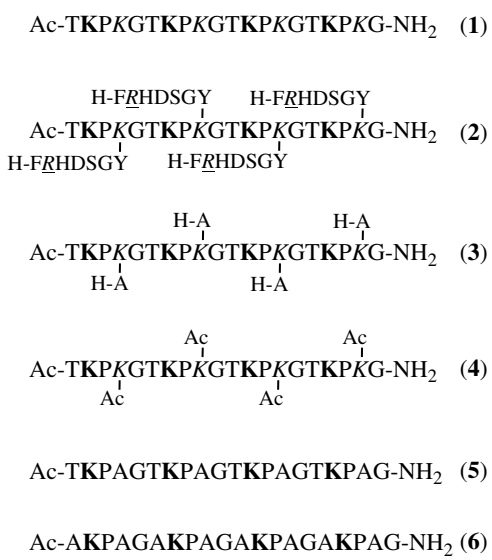

Figure 2 Schematic structures of oligotuftsin derivatives and related compounds.

the digest mixture did not significantly change at prolonged reaction times, and even after 24 h the same products were detected, indicating that no further cleavage had occurred (Figure 3(C)). These results are in agreement with previously reported data regarding the specificity of trypsin, confirming that trypsin cleaves specifically peptide bonds at the *C*-terminal side of lysine residues, but fails with Pro in the P′1 position.

The attachment of ß-amyloid(4–10) peptide at the Lys-Gly residues of the oligotuftsin backbone (attachment sites Lys-4, -9, -14, -19) provided the conjugate (**2**) which exhibited increased stability of the peptide backbone in the presence of trypsin. In this conjugate with branched structure, rapid cleavage of -Arg-His- bonds in the Aß-epitope side chain was identified (Figure 4, Table 1), but no rapid cleavage in the backbone. The presence of the Aß-peptide side chain abolished the tryptic cleavage of -Lys-Gly- bonds; however, fragments corresponding to cleavage at the four -Lys-Pro- sites were identified by mass spectrometry and analytical RP-HPLC (Figure 4, Table 1). The first fragments resulting from -Lys-Pro- cleavage were detected after 3 h. Significant differences in the cleavage rates between the four -Lys-Pro- bonds were observed, with the peptide bond-^{17}Lys-^{18}Pro- cleaved with the highest relative rate. We investigated further this cleavage by tryptic digestion of the peptides **3** and **4** in which the ε-amino groups of Lys-4, Lys-9, Lys-14 and Lys-19 were modified by acetylation (**4**) or by attachment of a single alanine residue

Table 1 HPLC and mass spectrometric characterization of tryptic fragments of oligotuftsin derivative and its conjugate with the Aß(4–10) epitope peptide[a]

Sequence	$[M + H]^+$ calculated/ found	HPLC[d] R_t (min)
Ac-$[TKPKG]_4$-NH_2, (**1**)	2105.2922/2105.3095[b]	18.4
Ac-TKPK-OH	515.63/515.80[c]	15.8
H-GTKPK-OH	530.64/530.70[c]	7.9
Ac-$[TKPK(H\text{-}FRHDSGY)G]_4$-$NH_2$, (**2**)	5558.16/5557.80[c]	35.9
Ac-$[TKPK(H\text{-}FRHDSGY)G]_3$TKPK(H-HDSGY)G-$NH_2$	5254.79/5255.10[c]	34.9
Ac-$[TKPK(H\text{-}FRHDSGY)G]_2$$[TKPK(H\text{-}HDSGY)G]_2$-$NH_2$	4951.42/4951.90[c]	33.5
Ac-$[TKPK(H\text{-}FRHDSGY)G][TKPK(H\text{-}HDSGY)G]_3$-$NH_2$	4645.2725/4645.3280[b]	32.1
Ac-$[TKPK(H\text{-}HDSGY)G]_4$-$NH_2$	4342.90032/4342.9432[b]	30.3
Ac-$[TKPK(H\text{-}HDSGY)G]_2$TK-OH	2431.2004/2431.2411[b]	27.5
Ac-$[TKPK(H\text{-}HDSGY)G]_3$TK-OH	3501.7150/3501.7147[b]	29.1
H-PK(H-HDSGY)G-NH_2	859.4061/859.4177[b]	20.8
H-PK(H-HDSGY)GTKPK(H-HDSGY)GTK-OH	2159.4932/2159.4390[b]	26.7
H-PK(H-HDSGY)GTKPK(H-HDSGY)G-NH_2	1929.9206/1929.9893[b]	26.7
H-PK(H-HDSGY)GTK-OH	1089.5328/1089.5572[b]	22.0
Ac-TKPK(H-HDSGY)GTK-OH	1360.6860/1360.7226[b]	23.7
H-PK(H-HDSGY)G$[TKPK(H\text{-}HDSGY)G]_2$-$NH_2$	3000.4351/3000.5879[b]	29.1
H-PK(H-FRHDSGY)G-NH_2	1162.5757/1162.6015[b]	26.7

[a] Incubation in 10 mM NH_4HCO_3 (pH = 8) solution at an enzyme to substrate ratio of 1 : 50 (w/w) at 37 °C.
[b] MALDI-FTICR mass spectrometric analyses were performed with a Bruker APEX II FTICR instrument equipped with an actively shielded 7 T superconducting magnet.
[c] MALDI-TOF mass spectra were recorded with a Bruker Biflex linear TOF mass spectrometer.
[d] Column: Nucleosil 300–7 C_{18} column (4 × 250 mm, 300 Å, 7 µm); Eluents: 0.1% TFA in water (A), 0.1% TFA in acetonitrile–water (80 : 20 v/v) (B); Gradient: 0 min 0% B; 5 min 0% B; 55 min 100% B; Flow rate 1 ml/min.

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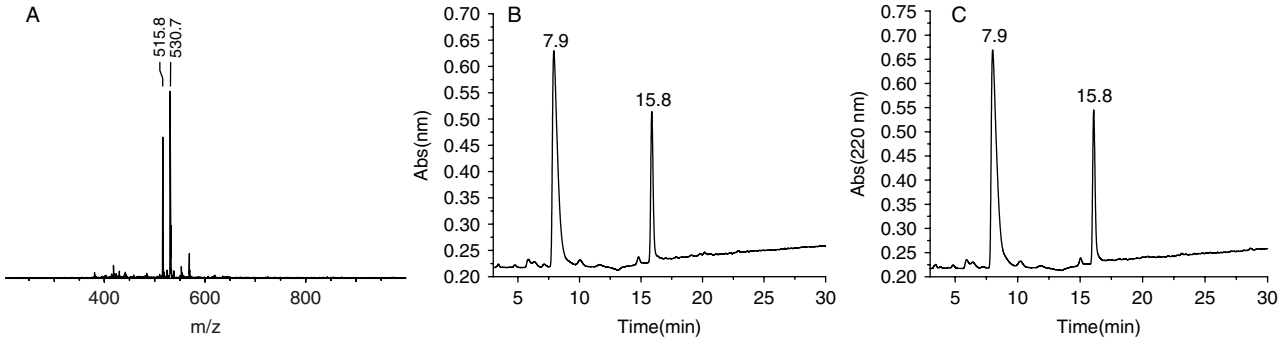

Figure 3 MALDI-TOF mass spectrum (A) and analytical HPLC profiles of oligotuftsin derivative (**1**) recorded after 15 min (B) and 24 h (C) incubation with trypsin. The two fragments correspond to H-GTKPK-OH (R_t = 7.9 min; 530.7 Da) and Ac-TKPK-OH (R_t = 15.8 min; 515.8 Da), respectively.

(**3**). MALDI-FTICR-MS and HPLC profiles showed the same -Lys-Pro- cleavage pattern, with the Lys-17-Pro-18 peptide bond being most susceptible to proteolysis (Figure 5, Table 2).

The substitution of Lys-4, Lys-9, Lys-14 and Lys-19 by alanine residues led to the peptide **5** in which all -Lys-Gly- bonds had been eliminated to evaluate the cleavage pathway of a linear peptide containing -Lys-Pro- bonds. All -Lys-Pro- bonds in this peptide were found to have approximately the same rate of cleavage by trypsin; in peptide **5** the Lys-17-Pro-18 peptide bond was not digested faster than the other peptide bonds. This peptide showed a relatively high stability toward trypsin digestion.

Kieliszewski and coworkers reported on the tryptic cleavage of -Lys-Pro- bonds in a hydroxyproline-rich glycoprotein from *Zea mays* [18]. In order to analyze whether the -OH side chains of threonine at the P2 positions had an effect on the degradation of the oligotuftsin peptides, both the Lys-Gly residues (Lys-4, -9, -14, -19) and threonine residues (Thr-1, -6, -11, -16) were replaced by alanine (peptide **6**). Interestingly, the -Lys-Pro- bonds of this peptide showed a drastically increased rate of hydrolysis by trypsin, with fragments corresponding to cleavages at all -Lys-Pro- bonds identified after 15 min. After 24 h of reaction, only the shortest peptide fragments were identified (Figure 6, Table 2), suggesting that the absence of hydroxyl groups and/or the bulky side chain of Thr residues provided a high rate of proteolysis of -Lys-Pro- bonds. To investigate a possible correlation between the secondary structures of the peptides and the differences in the degradation rates by trypsin, conformational preferences were determined by CD spectroscopy in 10 mM NH_4HCO_3 and in 100% TFE. In the aqueous buffer system used for tryptic digestion (10 mM NH_4HCO_3, pH 8), the peptides showed random coil conformation characterized by a strong negative π–π^* band around 198 nm; no significant differences between their conformations were detected. Deconvolution of the CD spectra using the least-square fit indicated that oligotuftsin

Table 2 Characteristics of fragments of side-chain- or backbone-modified oligotuftsin derivatives after digestion with trypsin[a]

Sequence	$[M+H]^+$ calculated/found	HPLC[d] R_t (min)
Ac-[TKPK(A)G]$_4$-NH_2, **3**	2389.4407/2389.4525[b]	18.4
Ac-[TKPK(A)G]$_3$TK-OH	2037.2185/2037.2279[b]	17.9
H-[PK(A)GTK]$_3$-OH	1454.8695/1454.8739[b]	17.2
Ac-[TKPK(A)G]$_2$TK-OH	1183.7163/1183.7142[b]	16.4
Ac-[TKPK(Ac)G]$_4$-NH_2, **4**	2273.3344/2273.3221[b]	19.9
Ac-[TKPK(Ac)G]$_3$-TK-OH	1950.1387/1950.1338[b]	19.2
H-[PK(Ac)GTK]$_3$-OH	1678.9015/1678.9867[b]	18.7
Ac-[TKPK(Ac)G]$_2$TK-OH	1396.8163/1396.8173[b]	18.2
H-[PK(Ac)GTK]$_2$-OH	1125.6631/1125.6597[b]	16.5
Ac-[TKPAG]$_4$-NH_2, **5**	1877.0608/1877.0257[b]	19.7
Ac-[TKPAG]$_3$TK-OH	1652.9335/1652.8995[b]	n.d.[e]
H-PAG[TKPAG]$_3$-NH_2	1605.9076/1605.8885[b]	n.d.
Ac-[TKPAG]$_2$TK-OH	1198.6752/1198.6674[b]	n.d.
H-PAGTKPAGTK-OH	927.5263/927.5184[b]	n.d.
H-PAGTKPAG-NH_2	697.3997/697.3921[b]	n.d.
Ac-[AKPAG]$_4$-NH_2, **6**	1758.07/1758.10[c]	18.2
Ac-[AKPAG]$_3$AK-OH	1533.81/1533.70[c]	15.7
H-PAG[AKPAG]$_3$-NH_2	1516.78/1516.60[c]	16.6
H-PAG[AKPAG]$_2$AK-OH	1292.52/1292.40[c]	17.0
Ac-[AKPAG]$_2$AK-OH	1109.31/1109.30[c]	17.0
H-PAG[AKPAG]$_2$-NH_2	1092.28/1092.40[c]	n.d.
H-PAGAKPAGAK-OH	868.02/868.50[c]	17.8
Ac-AKPAGAK-OH	684.81/685.10[c]	n.d.
H-PAGAKPAG-NH_2	667.78/668.20[c]	n.d.

[a] Incubation in 10 mM NH_4HCO_3 (pH = 8) solution at an enzyme to substrate ratio of 1 : 50 (w/w) at 37 °C.
[b] MALDI-FTICR mass spectrometric analyses were performed with a Bruker APEX II FTICR instrument equipped with an actively shielded 7 T superconducting magnet.
[c] MALDI-TOF mass spectra were recorded with a Bruker Biflex linear TOF mass spectrometer.
[d] Column: Nucleosil 300–7 C_{18} column (4 × 250 mm, 300 Å, 7 μm); Eluents: 0.1% TFA in water (A), 0.1% TFA in acetonitrile–water (80 : 20 v/v) (B); Gradient: 0 min 0% B; 5 min 0% B; 55 min 100% B; Flow rate 1 ml/min.
[e] Not determined because of the low amount of the compound.

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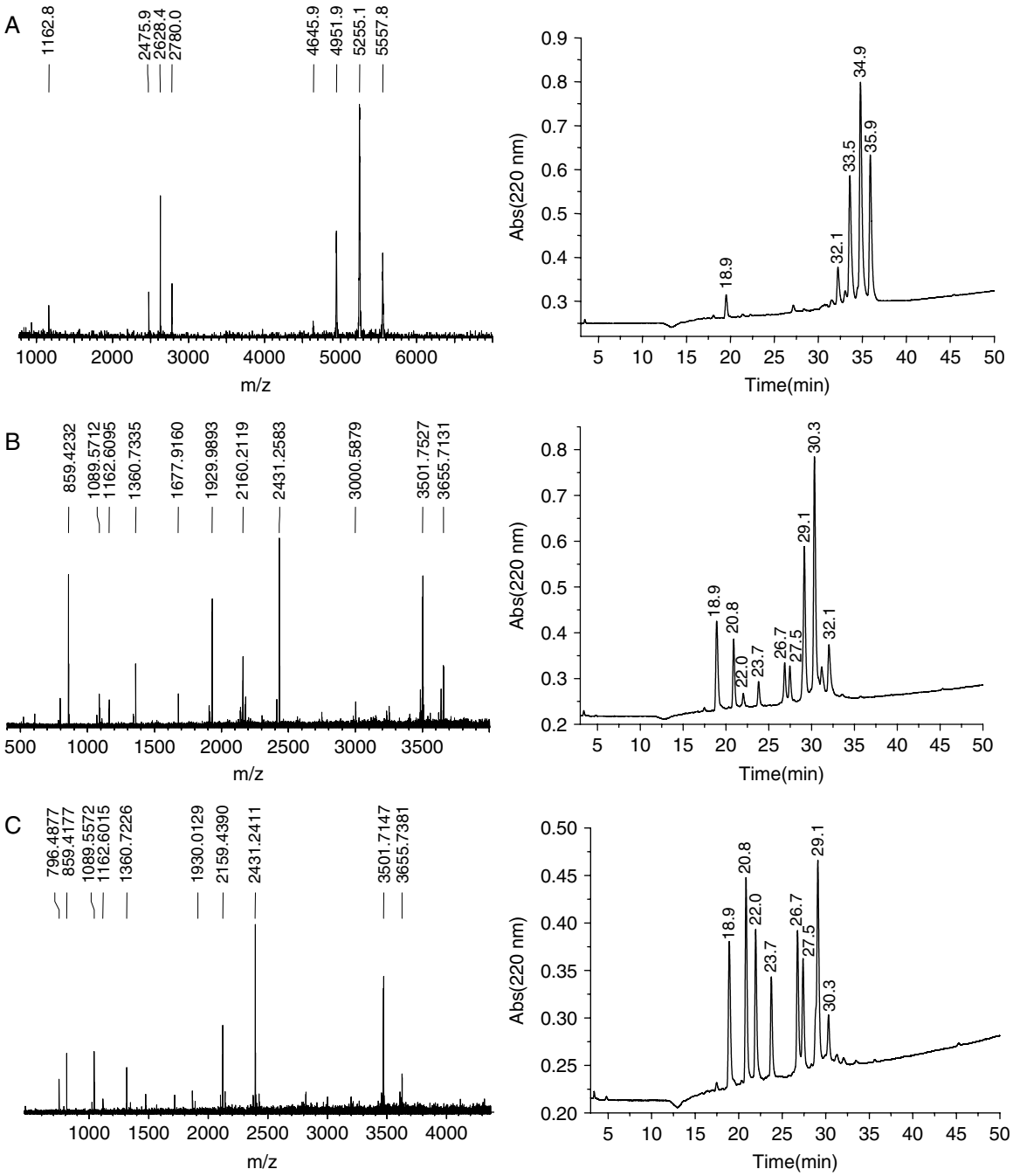

Figure 4 MALDI mass spectra and HPLC chromatograms of trypsin digests of ß-amyloid(4–10) epitope peptide conjugate with oligotuftsin derivative (**2**) after incubation with trypsin at an enzyme to substrate ratio of 1 : 50 (w/w) at 37 °C for 1 h (A), 9 h (B) and 24 h (C).

derivatives **1**, **3** and **4** adopt around 85% random coil conformation, whereas polypeptides **5** and **6** have 80% random coil conformation (data provided by the Fasman model). Differences between the secondary structures of the investigated compounds were observed in TFE, a solvent that preferentially stabilizes peptides and proteins in an ordered conformation [22]. The CD spectra were characterized by a negative band at 198 nm and a small negative shoulder at approximately 226 nm (Figure 7); the latter was more pronounced in the case of peptide **6** (Ac-$[AKPAG]_4$-NH_2). The CD spectra recorded in TFE reflect the predominance of an unordered structure (compounds **1**, **3** and **4** adopt approximately 70% random coil conformation, compound **5** has 68%, while oligotuftsin derivative **6** has 65% random coil conformation); however the percentage of the unordered structure is lower in comparison with that determined in aqueous solution. The content of α-helix calculated from the ellipticity indicated that in 10 mM NH_4HCO_3 approximately 2%

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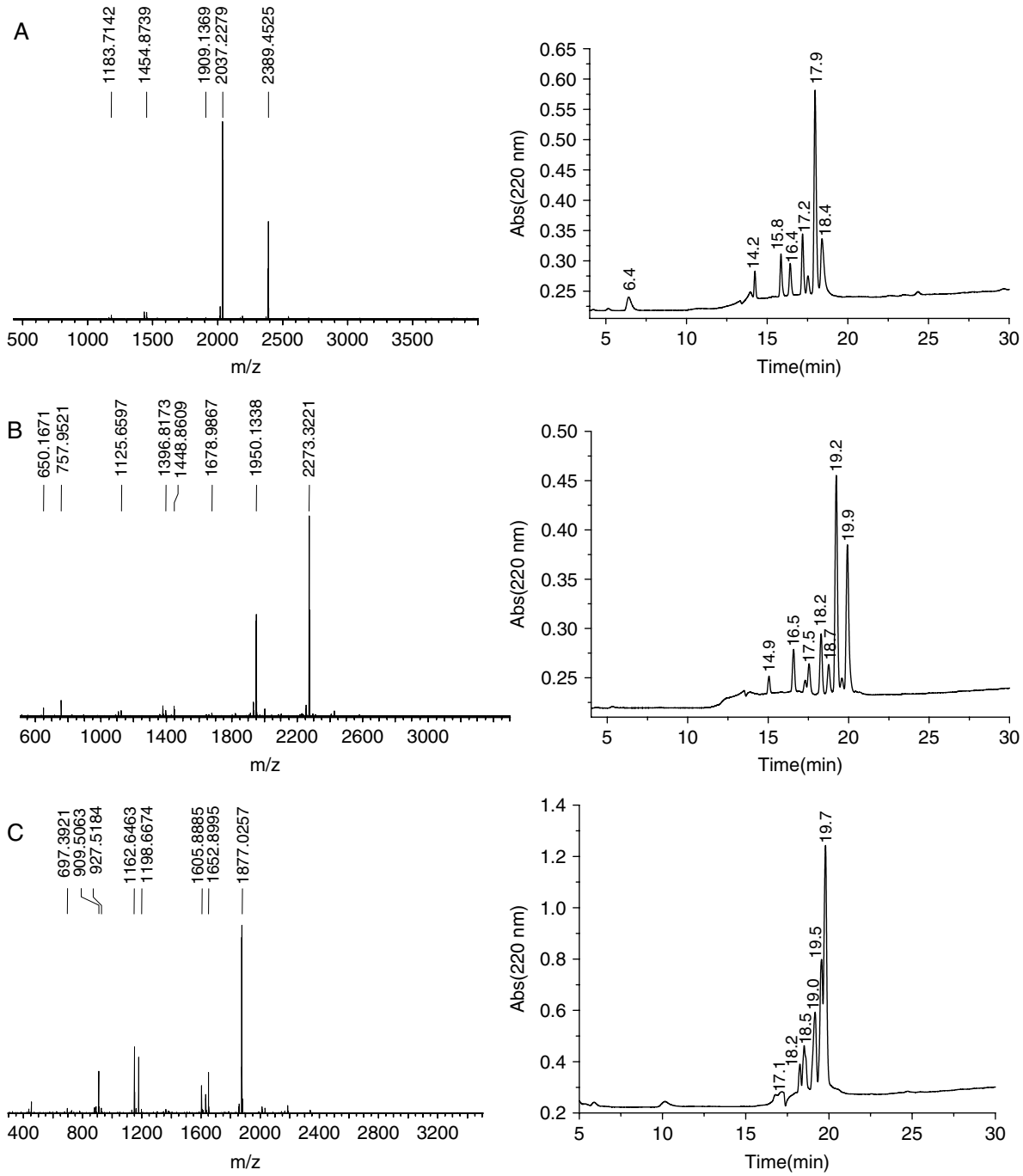

Figure 5 MALDI-FTICR mass spectra and HPLC profiles of modified oligotuftsin derivatives Ac-[TKPK(A)G]$_4$-NH_2 (**3**) (A), Ac-[TKPK(Ac)G]$_4$-NH_2 (**4**) (B) and Ac-[TKPAG]$_4$-NH_2 (**5**) (C) recorded after 24 h incubation with trypsin.

of the secondary structure elements were represented by the α-helix for all analyzed peptides. In TFE, for the oligotuftsin derivatives **1**, **3**, **4** and **5** the content of the α-helix structure was around 4–5%, whereas for **6** it was determined as 8%. The ability of compound **6** to undergo some conformational changes and to adopt more ordered structure in TFE might be associated with the increased flexibility of the peptide bonds.

To obtain further structural information about the oligotuftsin derivatives, a molecular modeling using the Hyperchem 7.0 program was performed. AMBER96 parameter set was applied for the geometry optimization, and calculations were performed by allowing the presence of water molecules. According to the molecular models (Figure 8), there are no pronounced differences between the structures of oligotuftsin derivatives; however, Ac-AKPAGAKPAG-NH_2 has a more flexible backbone promoting an open structure, this alanine-modified oligotuftsin showing a significantly higher accessibility to trypsin.

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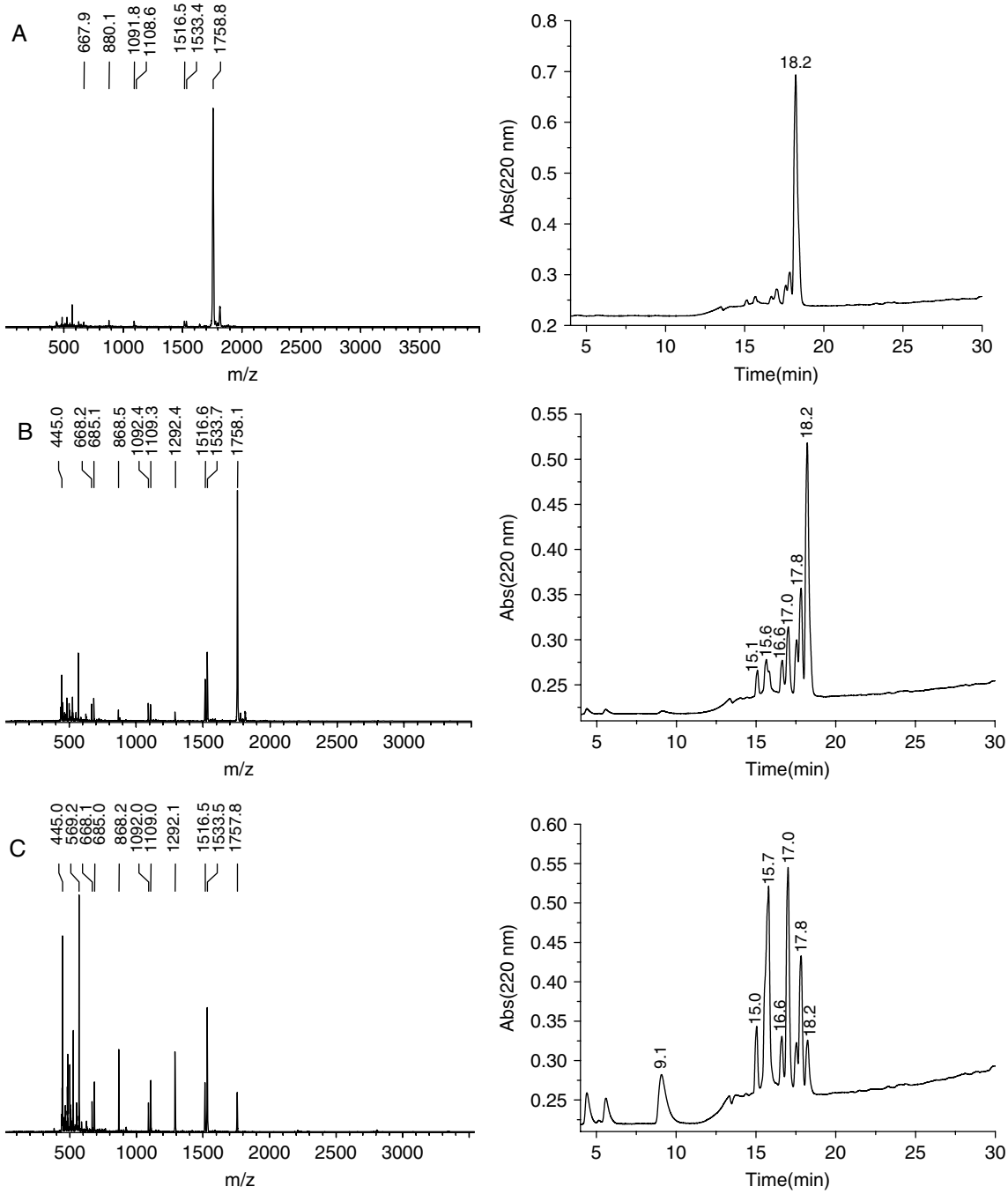

Figure 6 Trypsin digestion of modified oligotuftsin derivative Ac-[AKPAG]$_4$-NH$_2$ (**6**) followed by MALDI-TOF mass spectrometry and analytical RP-HPLC. Incubation time 15 min (A), 3 h (B) and 9 h (C).

CONCLUSIONS

In the present study we provide evidence for a -Lys-Pro- proteolytic cleavage by trypsin in modified oligotuftsin derivatives. The susceptibility of the -Lys-Pro- peptide bonds was found to be strongly dependent on the adjacent peptide sequence, and flexibility of the peptide backbone is suggested as a prerequisite for this cleavage. While the -Lys-Gly- bonds are shown to be rapidly cleaved by trypsin in the original tetratuftsin peptide, the substitution of the side chains of these Lys residues to yield -Lys(X)-Gly- derivatives prevents cleavage of this bond. However, during prolonged digestion with trypsin, cleavage of -Lys-Pro- bonds was additionally observed, the pathway of this degradation being independent on the type of Lys-N^{ε}-substituent (acetyl group, amino acid, peptide). Modification of the Lys-Gly residues by Ala at the P′2 positions generally decreased the tryptic cleavage, while removal and replacement of the bulky side chain of Thr at the P2 positions increased the proteolytic cleavage of the -Lys-Pro- bonds of the peptide backbone. These results

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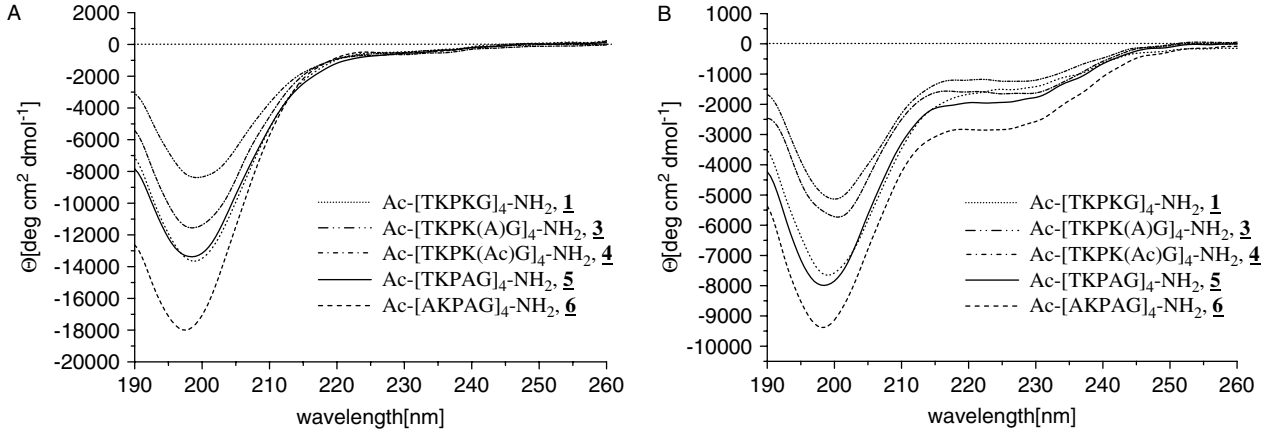

Figure 7 CD spectra of oligotuftsin derivative (**1**) and its side-chain modified (**3**), (**4**) and backbone-modified derivatives (**5**), (**6**). The spectra were recorded in 10 mM NH_4HCO_3, pH = 8 (A) and in 100% TFE (B).

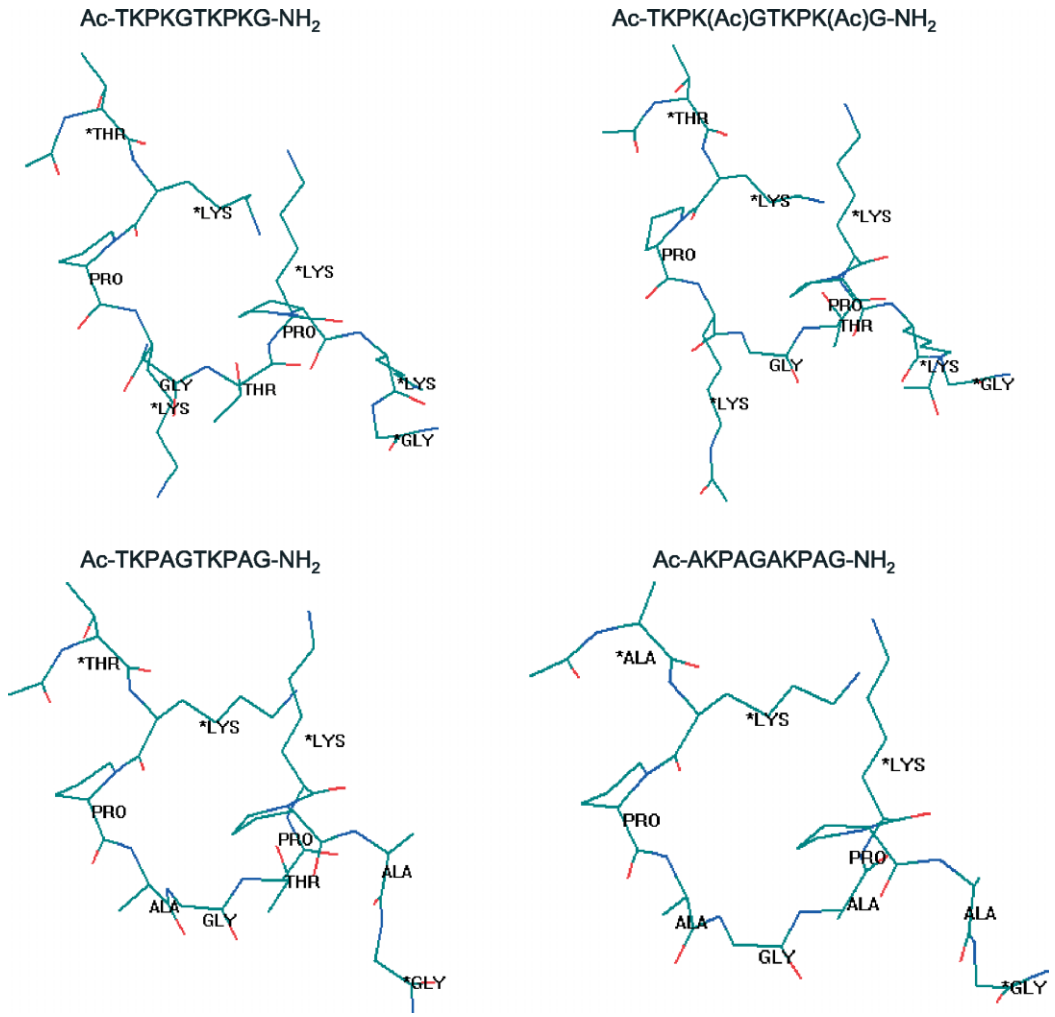

Figure 8 Molecular models of oligotuftsin derivatives.

corroborate well with the flexibility of the peptide backbone, rendering increased susceptibility to cleavage of -Lys-Pro- bonds. Furthermore, these results might have implications for proteolytic degradation studies that require specific conformational preconditions.

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