Proteolytic stability of β-peptide bonds probed using quenched fluorescent substrates incorporating a hemoglobin cleavage site

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Abstract A set of designed internally quenched fluorescence peptide substrates has been used to probe the effects of insertion of β-peptide bonds into peptide sequences. The test sequence chosen corresponds to a proteolytically susceptible site in hemoglobin α-chain, residues 32–37. Fluorescence and mass spectral measurements demonstrate that the insertion of an β-residues at the potential cleavage sites completely abolishes the action of proteases; in addition, the rate of cleavage of the peptide bond preceding the site of modification is also considerably reduced.

Key words: β-Peptides; Fluorescent protease substrate; Fluorescence resonance energy transfer; Mass spectrometry; Proteolytic stability; Hemoglobin

1. Introduction

Peptides composed of β-amino acid residues have been reported to be resistant to degradation by a variety of proteases. Extensive work from the laboratories of Dieter Seebach and collaborators has established the proteolytic stability of diverse β-oligopeptide sequences, suggesting that β-peptides are not readily acted upon by most commonly available proteases [1–5]. Considerable recent interest in the chemistry and biology of peptides containing β-amino acids stems from the potential utility of β-residues in generating proteolytically stable analogs of biologically active peptides [6–13]. In order to explore the effects of selective insertion of β-residues at proteolytically susceptible segments in peptide sequences, we have chosen to investigate a sequence derived from hemoglobin, α-chain residues 30–37 (Fig. 1), which is the site of initial attack by the proteases plasmepsin I and II, produced by the malarial parasite *Plasmodium falciparum* [14,15]. The strategy employed has been to synthesize a set of internally quenched, fluorescent substrates, with introduction of the β-residues at the cleavage site. The 4-(4-dimethylaminophenylazo)-benzoyl (DABCYL) and [*N*(-acetamidoethyl)]-1-naphthylamine-5-sulfonic acid (AEDANS) groups are employed as the intramolecular fluorescent energy transfer pair, with the former serving as the quencher and the latter as the fluorescent chromophore. Proteolysis of substrates results in a large increase of fluorescence at 490 nm, upon excitation at 335 nm. Quenched fluorescent substrates are widely used for the assay of proteases, ever since their development for the assay of retroviral proteases [16], and have been employed for the kinetic analysis of the activity of the malarial plasmepsins [17]. In the design of substrates listed in Fig. 1, we have used a cysteine residue at the C-terminus of the substrates to facilitate attachment of the AEDANS chromophore, using the readily available sulfhydryl reagent *N*-(iodoacetamidoethyl)-1-naphthylamine-5-sulfonic acid (I-AEDANS).

2. Materials and methods

2.1. General procedure for the synthesis of peptides

The peptides were synthesized by standard solid phase peptide synthetic methods using Fmoc chemistry [18]. All amino acids are protected at the N-terminus with the Fmoc group (NovaBiochem). The side chains of Glu and Ser were protected with the t-Bu group, Arg with the Mtr group, Cys with the trityl group and Lys with the Boc group. The Fmoc protected β-amino acids were synthesized by using published procedures [19]. The coupling reactions proceeded using the OPfp esters of the protected amino acids on Fmoc-PAL-PEG-PS resin (PerSeptive Biosystems). The synthesis was performed with 500 mg of resin with a bead capacity of 0.22 mmol/g. The C-terminal amino acid (Lys) was linked to the resin by the formation of an amide linkage. All β-amino acids, Ser and DABCYL group were coupled to the peptides by using HBTU (N-[(1H-benzotriazol-1-yl) (dimethylamino)-methylene]-*N*-methylenemethanaminium hexafluorophosphate *N*-oxide). The Fmoc deprotections were performed with 20% piperidine in dimethylformamide. Peptide was cleaved from 100 mg of resin after synthesis, using 94% TFA (7.52 ml), containing 5% anisole(400 μl) and 1% ethanedithiol (80 μl) as cation scavengers.

The isolated crude peptide (10 mg, 0.0064 mmol) was dissolved in a 8:2 mixture of THF and water (8 ml). Solid I-AEDANS (8.35 mg, 0.0192 mmol) was added to the solution. The The pH of the solution was adjusted to 8 by using triethylamine. The reaction mixture was stirred at room temperature for about 3 h. The solvent was evaporated in vacuo and the crude peptide was dissolved in a minimum amount of methanol. The purification of peptides was achieved by a reverse-phase HPLC (Hewlett Packard series 1100), on a C_{18} column, using a linear gradient of acetonitrile–water containing 0.1% TFA. The purified peptides were characterized by MALDI-TOF mass spectrometry.

I, $M_{obs} = 1984.0$, $M_{calc.} = 1984.70$; II, $M_{obs} = 1998.5$, $M_{calc.} = 1998.7$; III, $M_{obs} = 1998.2$, $M_{calc.} = 1998.7$; IV, $M_{obs} = 2013.0$, $M_{calc} = 2012.7$.

2.2. Enzyme assay

Plasmepsin II was obtained as a 42 kDa pro-protease in the form of insoluble, inclusion bodies from an expression construct in vector pET 23d. The insoluble protein was solubilized in urea purified and refolded using the protocol of Hill et al. [20]. Refolded pro-plasmepsin II was again purified and activated to obtain the mature protease by

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lowering the pH of the solution from 8.0 to 4.8. The mature plasmepsin II purified on Sephadex G-75, to remove the pro-peptide, was used in the proteolytic analysis. Proteases: α-chymotrypsin (Sigma) from bovine pancreas, trypsin (Sigma) from bovine pancreas, pepsin (Sigma) from porcine stomach and proteinase K (Boehringer Mannheim) from *Tritirachium album* were used for the cleavage assay. Fluorescence was monitored using a Perkin Elmer LS55B or Hitachi 650-60 spectrofluorimeters. Excitation and emission wavelengths were 335 and 490 nm, respectively. The slit widths were fixed at 10 nm each. Assays were conducted at room temperature in a total volume of 600 μl. 1.8 μM substrate was used for each reaction. The assay for plasmepsin II was performed in 100 mM sodium acetate buffer, pH 4.8. Protein concentration was measured by the Bradford method [21], bovine serum albumin being the standard. The concentration of the protein was adjusted to 75 μg/ml. One μl of this solution was added to 600 μl of assay mixture and increase in intensity of fluorescence was monitored. The assays of α-chymotrypsin, trypsin and proteinase K were performed in 100 mM triethanolamine buffer, pH 7.5. In the case of pepsin, the assay was conducted in 66 mM citrate–phosphate buffer, pH 3.0. One μl of 1 mg/ml stock solution of each protease was used to initiate the reaction. Increase in fluorescence intensity due to the hydrolysis of substrates was monitored at 1 min intervals until no further increase in fluorescence intensity was observed. The same reaction mixture was used for mass spectral analysis. MALDI spectra were recorded on a KOMPACT SEQ spectrometer, kratos analytical, Manchester, UK. The matrices used for positive ion mode detection were α-cyano-4-hydroxycinnamic acid and 3,5-dimethoxy-4-hydroxycinnamic acid in 60% acetonitrile containing 0.1% TFA. α-Cyano-4-hydroxycinnamic acid and 3,5-dimethoxy-4-hydroxycinnamic acid in 60% acetonitrile, along with 1% solution of ammonium acetate were used in the negative ion mode. Routinely, 0.5 μl of matrix was mixed with 0.5 μl of the reaction solution on a MALDI plate for mass spectral analysis.

3. Results and discussion

Fig. 2 shows the effect of addition of a solution of the *P. falciparum* aspartic protease, plasmepsin II. Substrate I, which incorporates the native α-chain of hemoglobin is rapidly cleaved, resulting in a strong fluorescence at 490 nm. Analysis of the cleavage products using MALDI mass spectrometry revealed the presence of peptide fragments with masses of 918 Da (fragment 1–5, positive ion mode) and 1082 Da (fragment 6–12, negative ion mode), corresponding to the cleavage of the peptide bond between Phe(5) and Leu(6). Indeed, earlier studies have established the Phe(33)–Leu(34) peptide bond as the preferred site of plasmepsin II cleavage of hemoglobin [14,15]. The effect of the addition of plasmepsin II to the substrates II, III and IV is also shown in Fig. 2. Clearly, all these substrates are resistant to proteolytic cleavage by plasmepsin II, suggesting that substitution of a β-residue at either the Phe or Leu position imparts resistance to peptide bond cleavage.

In order to further probe the consequences of β-residue insertion, we tested the cleavage of substrates I–IV with the proteases, chymotrypsin, trypsin, pepsin, and proteinase K. Fig. 3 illustrates the effect of the addition of chymotrypsin to solutions of the four fluorescent substrates. Table 1 summarizes the results of MALDI mass spectrometry analysis of the cleavage products obtained in the case of various enzymes. In the case of chymotrypsin, the cleavage of substrate I is facile, with the breakage of both Phe(5)–Leu(6) and Leu(6)–Ser(7) bonds. The insertion of β-HPhe in substrate II results in abolition of the cleavage of the β-HPhe–Leu bond. However, from Fig. 3 it is evident that there is a slow increase in fluorescence corresponding to cleavage of the Leu(6)–Ser(7) bond (Table 1). Interestingly, in Substrate III the rate of cleavage as monitored by fluorescence is extremely slow, with the MALDI analysis revealing that the site of cleavage is the Met(4)–Phe(5) bond. Substrate IV, which contains β-HPhe(5)–βLeu(6) segment, is completely resistant to cleavage by chymotrypsin, an enzyme with a reported specificity

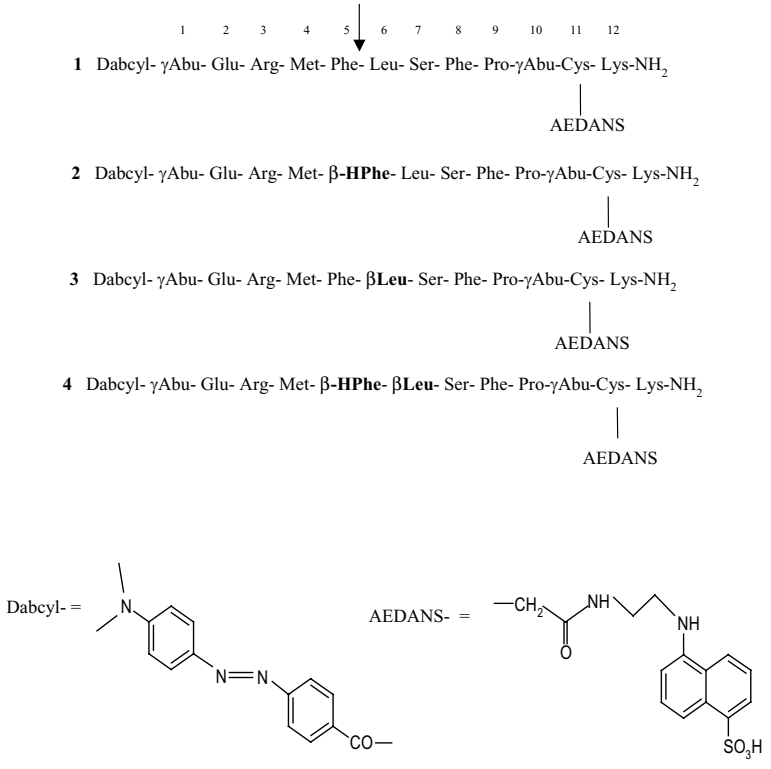

Fig. 1. Structures of internally quenched protease substrates. β^3-(*S*)-homophenylalanine is abbreviated as β-HPhe and β^3-(*S*)-homoleucine(βLeu) as βLeu for simplicity in this paper. The substitution is at position 3 of the 3-amino-propionyl moiety.

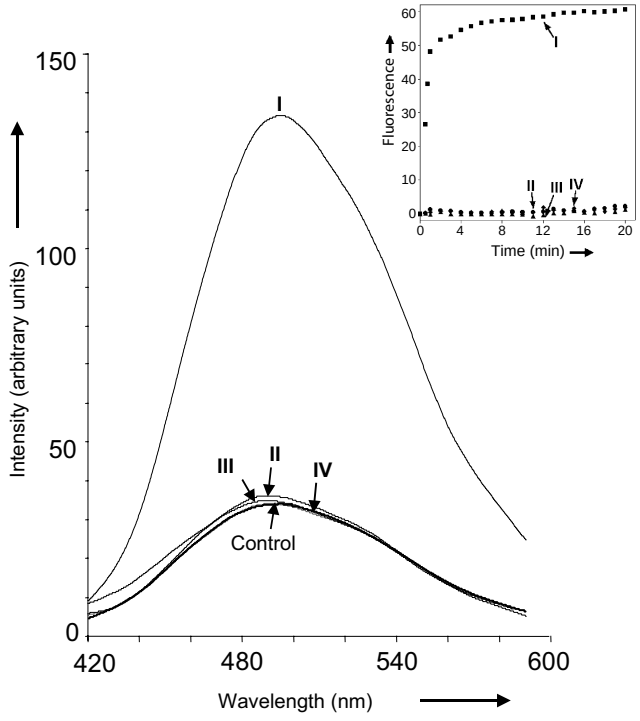

Fig. 2. Uncorrected fluorescence emission spectrum of I, II, III, and IV after the addition of plasmepsin II. Control 1.8 μM substrate in 100 mM sodium acetate buffer, pH 4.8. Traces I–IV correspond to the fluorescence emission spectra 1 min after the addition of plasmepsin II to the substrates. Excitation, 335 nm. The inset shows the time course of the fluorescence change after enzyme addition.

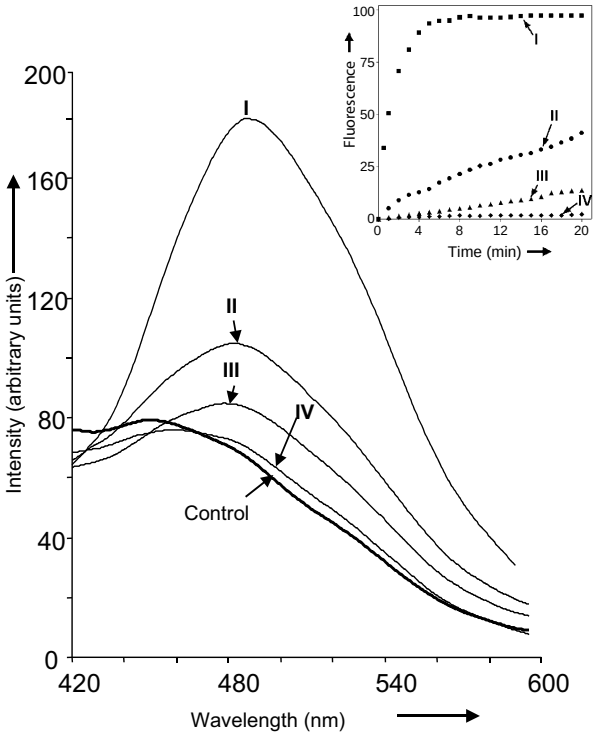

Fig. 3. Uncorrected fluorescence emission spectrum of I, II, III, and IV after the addition of chymotrypsin. Control 1.8 μM substrate in 100 mM triethanolamine buffer, pH 7.5. Traces I–IV correspond to the fluorescence emission spectrum 1 min after the addition of chymotrypsin to the substrates. Excitation, 335 nm. The inset shows the time course of the fluorescence change after enzyme addition.

for aromatic and large hydrophobic residues [22]. In the case of the chosen test sequences, the enzymes appear to be able to cut primarily at the segment between residues 4–6. The results suggest that the replacement of an α-amino acid by a β-residue at the specific cleavage site can confer proteolytic stability to the peptide. In addition, analysis of the rates of cleavage of substrate II and substrate III suggest that the β-residue insertion also retards the cleavage of the preceding peptide bond.

Substrate I is also readily cleaved by the enzymes pepsin (Met(4)–Phe(5), Phe(5)–Leu(6)), proteinase K (Phe(5)–Leu(6)) and trypsin (Arg(3)–Met(4)). Insertion of β-HPhe at position 5 abolishes the cleavage by pepsin and proteinase K (Table 1).

In substrate III, where residue 6 is βLeu, cleavage of the Met–Phe bond is observed in both pepsin and proteinase K. These results further confirm the effect of β-residues in protecting the peptide bond at the site of insertion and the preceding peptide bond from proteolysis. In all four substrates, cleavage by trypsin at the Arg(3)–Met (4) bond is observed. We determined kinetic parameters for hydrolysis of substrate I and substrate II by chymotrypsin: K_m, 9.4 mM for substrate I and 20.2 mM for substrate II; k_{cat}, 68 min^{-1} for substrate I and 30 min^{-1} for substrate II. The insertion of β-HPhe at position 5 abolishes the cleavage of the Phe(5)–Leu(6) bond

Table 1
Masses of peptide fragments after proteolytic cleavage[a]

Proteases	Substrate I		Substrate II		Substrate III	
	Positive ion mode	Negative ion mode	Positive ion mode	Negative ion mode	Positive ion mode	Negative ion mode
Plasmepsin II	918.7 (1–5)	919 (1–5), 1082.8 (6–12)	–[b]	–[b]	–[b]	–[b]
Chymotrysin	916 (1–5), 1031.6 (1–6)	919.3 (1–5), 1082 (6–12) 1032 (1–6), 971.6 (7–12)	1044.6 (1–6)	1045.3 (1–6), 971.3 (7–12)	771.3 (1–4)	772 (1–4), 1245.6 (5–12)
Pepsin	772.3 (1–4), 919.4 (1–5)	771.1 (1–4), 918.0 (1–5), 1230.8 (5–12), 1083 (6–12)	–[b]	–[b]	771.3 (1–4)	1245 (5–12)
Proteinase K	919 (1–5)	919.0 (1–5), 972.0 (7–12)	–[b]	–[b]	771.4 (1–4)	–[c]
Trypsin	641 (1–3)	640 (1–3), 1362.6 (4–12), 1384.7 (4–12) [M+Na]	641 (1–3)	640 (1–3), 1376.4 (4–12), 1398.7 (4–12) [M+Na]	641 (1–3)	–[c]

[a]Substrate IV was not cleaved by plasmepsin II, chymotrypsin, pepsin, proteinase K. Trypsin cleaved the bond between Arg(3) and Met(4) yielding a positive ion mass of 641 Da, corresponding to the 1–3 fragment.
[b]No cleavage observed.
[c]The C-terminus fragment was not detected.

and the measured rates correspond to the breakage of Leu(6)–Ser(7) bond. No inhibitory activity could be detected for substrates II, III, and IV in the case of the malarial enzyme, plasmepsin II.

The results obtained in this study clearly established the effect of insertion of β-residues on the proteolytic stability of a model α-peptide sequence. In the design of proteolytically stable analogs of biologically active peptides, knowledge of the most susceptible cleavage site may be of value in choosing positions for β-residue insertion.

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