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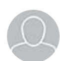
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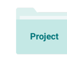
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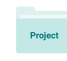
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Structure Formation in Short Designed Peptides Probed by Proteolytic Cleavage

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Abstract: The formation of local structure, in short peptides has been probed by examining cleavage patterns and rates of proteolysis of designed sequences with a high tendency to form β-hairpin structures. Three model sequences which bear fluorescence donor and acceptor groups have been investigated:

Dab-Gaba-Lys-Pro-Leu-Gly-Lys-Val-Xxx-Yyy-Glu-Val-Ala-Ala-Cys-Lys-NH$_2$

|

EDANS

Xxx-Yyy: Peptide **1**=^{D}**Pro**-^{L}**Pro**, Peptide **2**=^{D}**Pro**-Gly, Peptide **3**=**L**eu-Ala

Fluorescence resonance energy transfer (FRET) provides a convenient probe for peptide cleavage. MALDI mass spectrometry has been used to probe sites of cleavage and CD spectroscopy to access the overall backbone conformation using analog sequences, which lack strongly absorbing donor and acceptor groups. The proteases trypsin, subtilisin, collagenase, elastase, proteinase K and thermolysin were used for proteolysis and the rates of cleavage determined. Peptide **3** is the most susceptible to cleavage by all the enzymes except thermolysin, which cleaves all three peptides at comparable rates. Peptides **1** and **2** are completely resistant to the action of trypsin, suggesting that β-turn formation acts as a deterrent to proteolytic cleavage.

Keywords: Fluorescent protease substrate, fluorescence resonance energy transfer, mass spectrometry, circular dichroism, β-hairpin peptide, β- turn, proteases.

INTRODUCTION

The necessity to study proteases has led to the development of numerous assay methods. The use of internally quenched fluorescent substrates affords a very sensitive method for establishing proteolytic activity. The method was first introduced in the literature for the analysis of retroviral proteases [1] and has subsequently been used in several other cases like renin [2], hepatitis A3C protease [3], interleukin 1b converting enzyme (ICE) [4], *Leishmania* surface metalloprotease [5], anthrax lethal factor [6], *Candida albicans* aspartic protease [7], papain [8] and the plasmodial protease-plasmepsin II [9-11]. Earlier in our laboratory we have used this method to demonstrate the stabilization to proteolysis of a hemoglobinase cleavage site by plasmepsin and other proteases, upon incorporation of a beta-amino acid residue at the scissile site.

Following the success of the above approach, an internally quenched fluorescent 'universal' protease substrate, which contains several specific cleavage sites was designed. The principle of our approach is based on linearly assembling a sequence, which contains a large number of cleavage sites for well-characterized proteases. Since each cleavage site would be built into at least a tri or tetra peptide sequence, a substrate designed to be hydrolyzed by a variety of proteases will very quickly acquire a length of 25 residues or greater. The principle of internal quenching would then become less effective as the distance between the fluorescent group and the energy transfer acceptor exceeds 50Å. In order to obviate this difficulty we have chosen to insert the ^{D}Pro-Gly segment into the center of the designed sequence, as this segment has been previously demonstrated to be very effective in nucleating hairpin structures [12]. The use of the centrally positioned, chain reversal nucleus facilitates the coming together, in space, of fluorescent and quenching groups. In this synthetic substrate, proteolytic cleavage sites are laid out along the strand segments of a potential polypeptide β-hairpin. Fluorescence studies using this universal substrate, in combination with mass spectrometry, were used to profile the protease activities in a complex mixture like a *P. falciparum* cell extract in different stages of growth [13].

Though the use of ^{D}**Pro**-Gly segment permitted linear assembly of multiple cleavage sites with acceptable internal quenching, it did not provide the required total quenching of dansyl fluorescence in the universal protease substrate. The reduction of the fluorescence background in the substrate would be expected to enhance the sensitivity of assay. Therefore a new set of short peptides harboring the ^{D}**Pro**-Gly and ^{D}**Pro**-^{L}**Pro** segments were designed (Fig. **1**). These new substrates were designed to probe the conformation around the turn formed by ^{D}**Pro**-Gly and ^{D}**Pro**-^{L}**Pro** by incorporating a proteolytic cleavage site (trypsin) near the turn segment. The ^{D}**Pro**-^{L}**Pro** turn segments lead to greater hairpin stability. The studies reported here indicate that the use of proteases in

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combination with fluorescence and mass spectrometry is a sensitive tool to probe local structure in peptides.

MATERIALS AND METHODS

Materials

Chemicals used in solid phase synthetic procedures were obtained from Novabiochem (Nottingham, UK) or Sigma (St.Louis, Mo). Trifluroacetic acid (TFA) and piperidine were purchased from Chem-Impex International Inc. (Wood Dale, Il). IAEDANS (N-(iodoacetamidoethyl)-1-naphthyl-amine-5-sulfonicacid), and DABCYL (4-(4-dimethylamino-phenylazo)-benzoyl) were purchased from Sigma Chemical Co., St. Louis, U.S.A. All other chemicals used were from local manufacturers like Ranbaxy and E. Merck India.

Synthesis of Substrates

The peptides were synthesized by standard solid phase peptide synthetic methods using Fmoc (fluorenylmethoxy carbonyl) chemistry [14]. All amino acids are protected at the N-terminus with the Fmoc group (Novabiochem). The side chain of Glu was protected with a tertiary butyl (tBu) group, Cys with a triphenylmethyl (trityl) group and Lys with a tert-butyloxycarbonyl (Boc) group. The coupling reactions proceeded using the pentafluorophenyl (Opfp) esters of the protected amino acids on Rink amide resin (Novabiochem). 4-(4-dimethylaminophenylazo)-benzoyl (DABCYL) group was coupled to the peptides by using N-[1H-benzotriazole-1-yl) (dimethylamino)-methylene]–N-methyl-enemethanaminium hexafluorophosphate N-oxide (HBTU) / triethylamine / N-hydroxybenzotriazole (HOBt). The Fmoc deprotections were performed with 20% piperidine in dimethylformamide. The resin assembled peptide (25 mg) was subsequently cleaved in 94% trifluoroacetic acid (TFA) (1.88 ml), containing 5% anisole (100μl) and 1% ethanedithiol (20μl) as cation scavengers. The crude peptides were characterized before dansylation by MALDI-TOF mass spectrometry-peptide: Peptide **1**: M_{obs}=1770.4[MH^+], M_{calc}= 1769; Peptide **2**: M_{obs}=1729[M+H], M_{calc}=1728; Peptide **3**: M_{obs}=1761[M+H], M_{calc}=1760.

During the synthesis of the Peptide **1**, a deletion peptide was obtained and identified as a Val deletion peptide (next to the C-terminus of Lys residue) from mass spectrum analysis (M_{calc}= 1937; $MH+_{obs}$= 1940.1 assigned to deletion of Val; des-Val peptide). Attempts to separate the deletion peptide from the expected peptide were not successful. Hence all further derivatisation and fluorescence experiments were done using this mixture, which is estimated to contain ≈ 40% deletion peptide as judged by mass spectral intensities assuming equal ionization efficiencies.

Dansylation

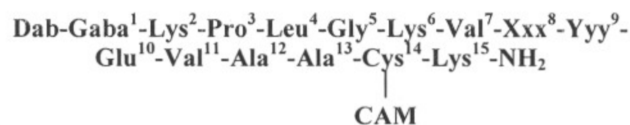
The isolated crude peptide (10 mg) was dissolved in 1M tris–HCl pH 8.0, N- (iodoacetamidoethyl)-1-naphthylamine-5-sulfonic acid (IAEDANS) (>5 equiv.) was added to the solution. The reaction mixture was kept at room temperature for about 3-4 hr. The crude peptide after labeling with EDANS was passed through a G-25 column using water as eluent, in order to remove excess 1-dimethylamino- 5-naphthalene sulfonic acid (DANS-OH). The fractions containing the peptide were pooled and concentrated. Further purification of peptides were achieved by reverse phase HPLC on a C_{18} column with a linear gradient of acetonitrile–water containing 0.1% TFA. The purified peptides thus obtained were free from contaminating fluorescencent by-products and thereby increasing the sensitivity of the assay. The purified peptides were characterized by MALDI-TOF mass spectrometry—peptide: Peptide **1**: M_{obs}=2078[M+H], M_{calc}=2077; Peptide **2**: M_{obs}=2037.7[MH^+], M_{calc}=2036; Peptide **3**: M_{obs}=2069.8 [MH^+], M_{calc}=2068.

FLUORESCENCE STUDIES

Fluorescence emission spectra were recorded on a Perkin Elmer LS55B or Hitachi 650-60 spectrofluorimeter or F-2500 spectrofluorimeter. The excitation wavelength was 336nm and the emission was scanned from 425-625nm, at different intervals of time. For time course studies the excitation was 336nm and increase in intensity was monitored at 490nm.

MALDI MASS SPECTROMETRY

Initial studies revealed suppression of ionization of the C-terminal fragment due to the dansyl group, presumably due to tight ion pair formation involving the sulfonic acid group. In order to increase the sensitivity of MALDI mass spectral analysis, the peptide after deprotection from the resin was labeled with iodoacetamide (IAM) (carboxamidomethylation) to block the free cysteine sulfhydryl group. The isolated crude peptide (10 mg) was dissolved in 1.5ml, 1M tris–HCl pH 8.0, iodoacetamide (> 3 equiv.) was added to the solution. The reaction mixture was kept at room temperature for about 4 hr. The substrates which contain a carboxamido group on cysteine with the sequence



Xxx-Yyy: Peptide **1a**=^{D}Pro-^{L}Pro, Peptide **2a**=^{D}Pro-Gly, Peptide **3a**= Leu-Ala, were then used for all mass spectral studies. The peptides purified by HPLC were characterized by MALDI-TOF mass spectrometry—peptide: Peptide **1a**: M_{obs}=1826.9[M+H], M_{calc}=1826; Peptide **2a**: M_{obs}=1786 [M+H], M_{calc}=1785; Peptide **3a**: M_{obs}=1840[$M+Na]^+$, M_{calc}=1817. Excellent MALDI -MS analysis could now be obtained, following proteolysis.

For recording MALDI spectra, the reaction volume was reduced to 50μl and the reaction stopped by freezing in liquid nitrogen. MALDI spectra were recorded in positive ion mode using a KOMPACT SEQ spectrometer (Kratos-Shimadzu analytical, Manchester, U.K.). The matrices used for positive ion mode detection were α-cyano-4-hydroxy-cinnamic acid and 3,5-dimethoxy-4-hydroxycinnamic acid in 60% acetonitrile containing 0.1% TFA. Routinely, 0.5μl of matrix was mixed with 0.5μl of the reaction solution on a MALDI plate for mass spectral analysis. Each sample was spotted thrice and spectra were recorded for each spot.

CIRCULAR DICHROISM

For circular dichroism studies it was necessary to examine peptides which did not contain the strongly UV absorbing groups DABCYL and EDANS. Consequently CD spectra were recorded for peptides in which the Fmoc deprotection and cleavage from resin were carried out after the addition of the Fmoc-Gaba residue. The peptides used for CD were purified by HPLC and characterized by MALDI mass spectrometry. Peptides used for CD had the following sequences:

Gaba-Lys-Pro-Leu-Gly-Lys-Val-Xxx-Yyy-Glu-Val-Ala-Ala-Cys-Lys-NH_2

Xxx-Yyy: Peptide 1b=^{D}Pro-^{L}Pro, Peptide 2b=^{D}Pro-Gly, Peptide 3b = Leu-Ala

Far UV CD spectra were recorded on a JASCO 715 spectropolarimeter equipped with a thermostatted cell holder. All the spectra were smoothened and baseline corrected. Cells of path length 1mm were employed. Spectra were averaged over 4 scans at a scan speed of 20nm/min. The spectra were recorded in both aqueous as well as in organic solvents.

ENZYME ASSAYS

Trypsin (Sigma) from bovine pancreas, elastase (Sigma) from porcine pancreas, collagenase (Sigma) from *Clostridium histolyticum,* subtilisin (Sigma) from *Bacillus licheniformis*, thermolysin (Sigma) from *Bacillus thermoproteolyticus* and proteinase K (Boehringer, Mannheim) from *Tritirachium album* were used for the cleavage assay. Fluorescence was monitored using a F-2500 spectrofluorimeter (Hitachi). Excitation and emission wavelengths were 336 and 490nm, respectively. The band pass was fixed at 5 or 10nm. Assays were carried out in triethanolamine buffer (TEA) pH 8 at room temperature. Typically, the assay contained 279μl of TEA buffer 100mM pH 8, having substrate concentration of 1.2μM. The reaction was initiated by adding 1μl of 1mg/ml stock solution of proteinase K, trypsin, subtilisin, while 5μl of 1mg/ml stock solution of thermolysin and elastase was used. The collagenase used for the study was 10μl of 1mg/ml stock solution. The same reaction mixture was used for mass spectral analysis.

RESULTS AND DISCUSSION

Short peptides harboring the ^{D}**Pro-Gly** and ^{D}**Pro-**^{L}**Pro** segments were designed as shown in (Fig. **1**). The insertion of a central ^{D}**Pro-**(Xxx) segment has been shown to facilitate β-hairpin formation in short peptides by nucleating type II' or type I' β turn structures [12,15-19]. ^{D}**Pro-**^{L}**Pro** segments readily form type II' β turns [20] and have been used to nucleate β hairpins in designed cyclic peptides [21]. The use of **L-Pro** at the i+2 position of the turn limits conformational flexibility as compared to the Gly peptide.

These new substrates were designed to probe the conformation around the turn formed by ^{D}**Pro-**^{L}**Pro** and ^{D}**Pro-**Gly by incorporating a proteolytic cleavage site (trypsin) near the turn segment. This approach is based on the fact that structured regions such as helices, beta sheets, and tight turns are anticipated to be more resistant to cleavage by proteases, as compared to dynamic non-structured regions. Peptide **3** which has a central **Leu-Ala** segment, serves as a control sequence, since structure formation is likely to be much less favored.

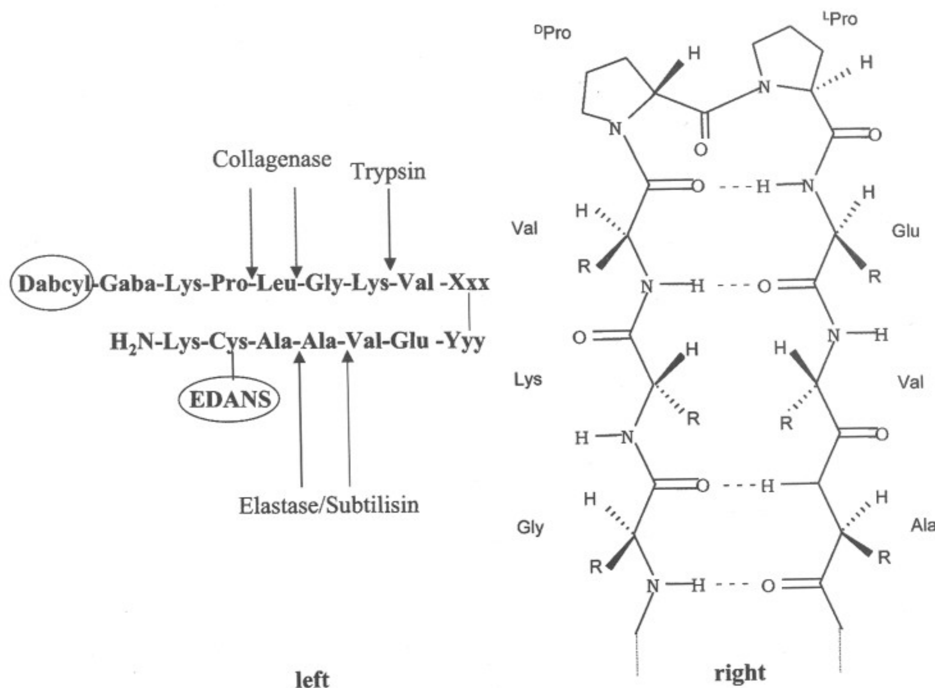


Figure 1. (left) Sequence of designed internally quenched fluorescence protease substrates Xxx = ^{D}**Pro** and **Y**xx = ^{L}Pro (Peptide **1**); Xxx = ^{D}**Pro** and **Y**xx = Gly (Peptide **2**); Xxx = **Leu** and **Y**xx = Ala (**Peptide 3**); **(right)** Schematic representation of hairpin conformation of peptide **1**.

The three designed peptides were investigated for their cleavage efficiency with different proteases. Fig. 2 shows the rates of cleavage of peptide **1**, peptide **2** and peptide **3**. From (Fig. 2) it is clear that the peptide **3** is more susceptible to cleavage by different proteases, than the peptides **1** and **2**. In the case of thermolysin, cleavage rates are comparable for all the peptides. Peptides **1** and **2** show complete resistance to trypsin cleavage.

The designed peptides without the donor and acceptor groups, peptides **1b**, **2b** and **3b** were used for the CD studies, to probe the conformation of the peptides in solution (tris-HCl pH 8.0). Fig. **3** shows the CD spectrum for the peptides. CD bands characteristic of unfolded conformations are observed in all the three peptides (Fig. **3a**). Hence, to induce structure, the peptides were titrated with different concentrations of TFE, a known promoter of secondary structure in peptides and proteins [22-23]. Fig. **3b**) shows the CD spectrum of the peptides at ~ 25% TFE concentration. **Peptide 1b** shows a single negative band around 200nm, while the **2b** shows a negative band around 200nm and a small negative dip at 222nm indicating the unstructured nature of peptides even at ~ 25% TFE. **Peptide 3b** shows negative bands at ~200 and 222nm characteristic of short peptide helices, suggesting that structure formation in **3b** is more facile than in **1b** and **2b**. This may be due to the fact that the central ^{D}**Pro**-^{L}**Pro** and ^{D}**Pro**-Gly segments are nucleators of 'prime turns' and consequently disrupt continuous helix formation, whereas **P**eptide **3b** (**L**eu-Ala) has residues which have a propensity to adopt helical conformations.

Conformational constraints can be divided conceptually into three following categories: local constraints involve restricting the conformational mobility of a single residue in peptide; regional constraints are those which affect a group of residues that form some secondary structural unit, such as a helix or sheet; global constraints involve the entire peptide structure. The fluorescence and CD studies together indicate a local structure in the vicinity of the ^{D}**Pro**-Gly and ^{D}**Pro**-^{L}**Pro** segment.

Mass spectral analysis was carried out to study the cleavage sites of the peptides **1a-3a** and the results obtained are tabulated in Table **1**. The different rates observed for the cleavage of the peptides with trypsin as determined by the fluorescence experiments may be a result of structure formation or the presence of D-amino acid near the site of cleavage. Indeed, D-amino acids have been used to stabilize the peptide bond against protease action [24-26]. In the present case, this possibility can be ruled out as the D-amino acid

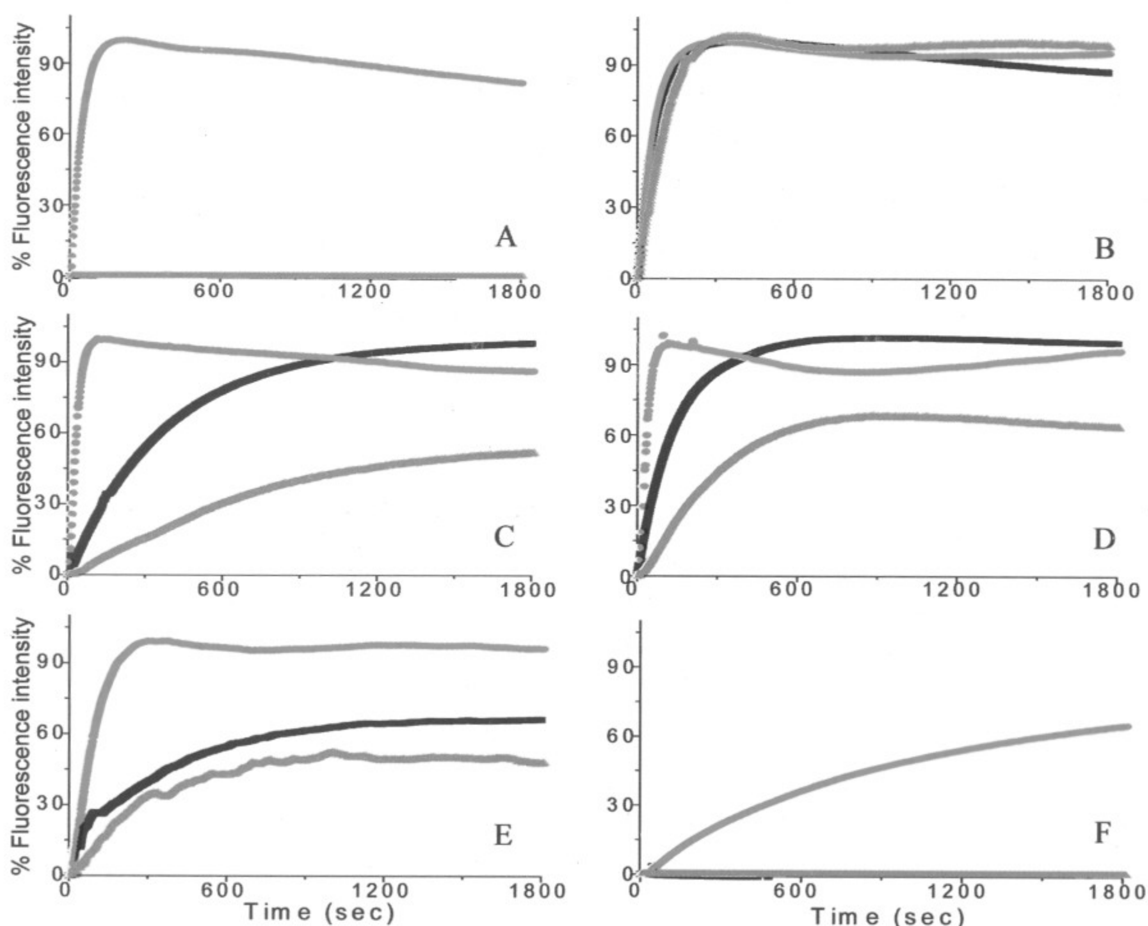


Figure 2. The time course of the fluorescence change after enzyme addition to solutions of designed peptides. A) trypsin, B) thermolysin, C) proteinase K, D) subtilisin, E) elastase, F) collagenase. Peptide **1** (▲), Peptide **2** (■) and Peptide **3** (●).

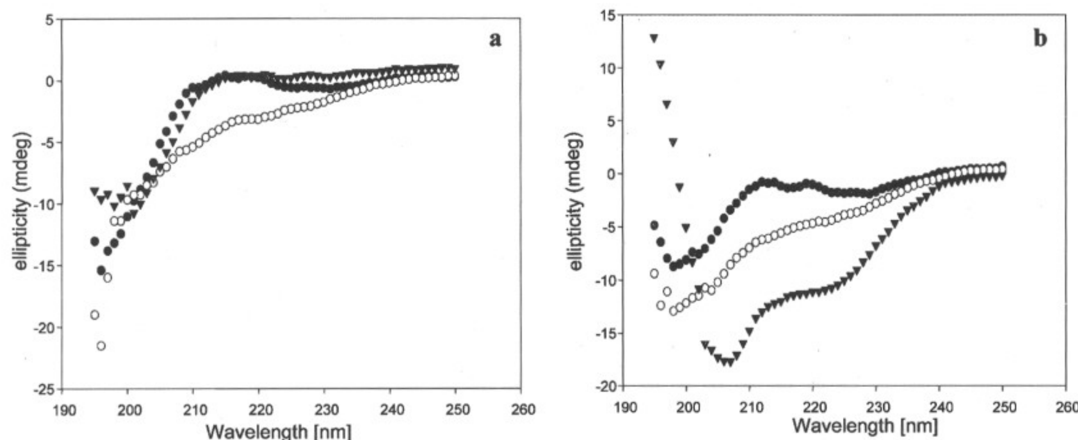


Figure 3. Far UV CD spectrum of peptides lacking donor and acceptor groups, **1b-3b**. A) Tris buffer pH 8.0; B) 25% trifluroethanol (TFE). **1b** (○), **2b** (●) and **3b** (▼).

Table 1. Masses of Peptide Fragments (Da) After Proteolytic Cleavage of 1a-3a, Observed by MALDI-MS

Enzyme	Peptide 1a			Peptide 2a			Peptide 3a		
	MH^+ observed	MH^+ calculated	Cleavage site	MH^+ observed	MH^+ calculated	Cleavage site	MH^+ observed	MH^+ calculated	Cleavage site
Trypsin	-	-	-	-	-	-	878(MH^+) 960(MH^+)	876 958	Lys^6-Val^7 Lys^6-Val^7
Collagenase	-	-	-	693(MH^+) 579(MH^+) 1267($M+K^+$)	691 578 1226	Leu^4-Gly^5 Pro^3-Leu^4 Pro^3-Leu^4	715($M+Na^+$) 1088(MH^+) 1145 (MH^+)	691 1086 1143	Leu^4-Gly^5 Gly^5-Lys^6 Leu^4-Gly^5
Subtilisin	487 ($M+K^+$) 548(MH^+) 1490($M+Na^+$) 1507($M+K^+$)	447 546 1468 1468	Val^{11}-Ala^{12} Glu^{10}-Val^{11} Ala^{12}-Ala^{13} Ala^{12}-Ala^{13}	568(M+Na) 1359(MH^+) 1452($M+Na^+$)	546 1357 1428	Glu^{10}-Val^{11} Val^{11}-Ala^{12} Ala^{12}-Ala^{13}	677(MH^+) 899($M+K^+$) 1090(MH^+) 1483($M+Na^+$) 998($M+Na^+$)	675 859 1088 1458 975	Ala^9-Glu^{10} Val^7-Leu^8 Leu^8-Ala^9 Ala^{12}-Ala^{13} Val^7-Leu^8
Elastase	449(MH^+) 1470(MH^+) 1399(MH^+)	447 1468 1397	Val^{11}-Ala^{12} Ala^{12}-Ala^{13} Val^{11}-Ala^{12}	588($M+K^+$) 1299 ($M+K^+$) 1430(MH^+) 1501(MH^+)	546 1258 1428 1499	Glu^{10}-Val^{11} Glu^{10}-Val^{11} Ala^{12}-Ala^{13} Ala^{13}-Cys^{14}	677(MH^+) 861(MH^+) 877(MH^+)	675 859 876	Ala^9-Glu^{10} Val^7-Leu^8 Lys^6-Val^7

-Differences in calculated and observed fragment masses arise due to errors of calibration.

residue, D-proline, is one amino acid away from the cleavage site of trypsin. According to the Schechter and Berger [27] nomenclature of the active site of proteases and their model for the interaction with substrate, it is considered that the amino acid residues of the polypeptide substrate bind at *subsites* of the active site. By convention, these subsites on the protease are called S (for subsites) and the substrate amino acid residues are called **P** (for peptide). The amino acid residues of the N-terminal side of the scissile bond are numbered **P**3, **P**2, **P**1 and those residues of the C-terminal side are numbered **P**1', **P**2', **P**3'.... The **P**1 or **P**1' residues are those residues located near the scissile bond. The substrate residues around the cleavage site can then be numbered up to P8. The subsites on the protease that complement the substrate binding residues are numbered S3, S2, S1, S1', S2', S3'... The residue at S2', S3'... may be unimportant compared to S1, S2, S3 for binding of the peptide near the active site as they make fewer interactions with protease, presumably, because this portion of the substrate is to be expelled first as a product [28,29].

Figure **4** shows the schematic subsites in the protease trypsin with the sequence of the peptide **2**. D-proline occupies the S2' subsite. Bovine pancreatic trypsin inhibitor (BPTI) a small inhibitor protein of 58 amino acids binds to trypsin through hydrogen bonding, forming a tightly packed interface between inhibitor and enzyme. The Michaelis-Menten constant of Bovine PancreaticTrypsin Inhibitor binding is $K_m = 10^{-13}$M. Lysine 15 binds to the specificity

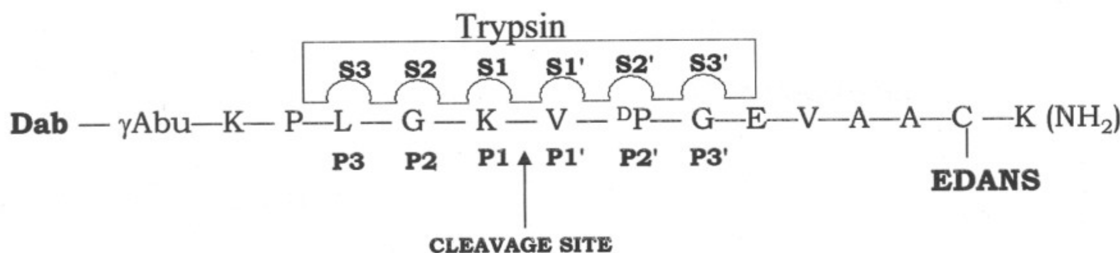


Figure 4. Schematic representation of interaction of peptide **2** with the trypsin. Schechter and Berger nomenclature is used to designate the peptide and the protease-binding sites. The binding subsites on the protease are called "S" and the substrate amino acid residues are called "P"

pocket followed by an alanine residue. The reaction is blocked at the formation of the transition state intermediate. Analyzing the structure of this complex, the C-terminus P2' residue is arginine (R17), which does not contribute much to the binding [29]. Hence, it is clear that S2' subsite is not a critical site and can accommodate any residue. In order to examine whether peptides **1** and **2** bind to trypsin, a competition assay was carried out using peptide **3** as the FRET substrate. The rate of cleavage of peptide **3** was monitored in the presence of peptides **1b** or **2b**, which lacked donor and acceptor groups. No differences were observed in the cleavage rates suggesting the absence of binding of **1b** and **2b** to trypsin.

The observed resistance to proteolytic attack by trypsin in case of **P**eptide **1** and **2** suggests that the vicinity around the ^{D}**P**ro-^{L}**P**ro and ^{D}**P**ro-Gly segment has formed a local structure. **P**eptide **3** is cut much more rapidly by trypsin than peptide **1** and **2**. The proteases subtilisin, thermolysin and proteinase K which show broad specificity have more or less comparable rates of cleavage, while specific proteases such as elastase and collagenase shows reduced rates with peptides **1** and **2**. The sites of attack of these proteases (elastase and collagenase) lie close to the ^{D}**P**ro-Gly and ^{D}**P**ro-^{L}**P**ro region. These results further support local structure formation in peptides **1** and **2**. Protease cleavage rates appear to be a sensitive tool in assaying structure formation in designed peptides.

ACKNOWLEDGEMENTS

This research was supported by the NMITLI project, Biotechnology for Leather, sponsored by the Council for Scientific and Industrial Research, Government of India.

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Received: June 03, 2005 Revised: October 24, 2005 Accepted: October 24, 2005

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