Bioorganic & Medicinal Chemistry 18 (2010) 8188–8193

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Implication of the disulfide bridge in trypsin inhibitor SFTI-1 in its interaction with serine proteinases

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ARTICLE INFO

Article history:
Received 20 May 2010
Revised 27 September 2010
Accepted 6 October 2010
Available online 29 October 2010

Keywords:
Cystein bridge
Proteinase inhibitor
Serine proteinase
SFTI-1

ABSTRACT

Fourteen monocyclic analogues of trypsin inhibitor SFTI-1 isolated from sunflower seeds were synthesized by the solid-phase method. The purpose of this work was to establish the role of a disulfide bridge present in inhibitor's side chains of Cys3 and Cys11 in association with serine proteinases. This cyclic fragment was replaced by the disulfide bridges formed by L-pencillamine (Pen), homo-L-cysteine (Hcy), *N*-sulfanylethylglycine (Nhcy) or combination of the three with Cys. As in the substrate specificity the P_1 position of the synthesized analogues Lys, Nlys [*N*-(4-aminobutyl)glycine], Phe or Nphe (*N*-benzylglycine) were present, and they were checked for trypsin and chymotrypsin inhibitory activity. The results clearly indicated that Pen and Nhcy were not acceptable at the position 3, yielding inactive analogues, whereas another residue (Cys11) could be substituted without any significant impact on the affinity towards proteinase. On the other hand, elongation of the Cys3 side chain by introduction of Hcy did not affect inhibitory activity, and an analogue with the Hcy–Hcy disulfide bridge was more than twice as effective as the reference compound ([Phe^5] SFTI-1) in inhibition of bovine α-chymotrypsin.

1. Introduction

The trypsin inhibitor SFTI-1 isolated in 1999 from the sunflower seeds by Luckett et al.[1] is currently the smallest naturally occurring peptidic proteinase inhibitor. This peptide displays the strongest trypsin inhibitory activity among Bowman-Birk family of inhibitors. The $P_1 - P'_1$ reactive site (according to the Schechter and Berger's notation)[2] of this cyclic inhibitor is located between residues Lys5 and Ser, the former being responsible for the specificity of the inhibitor. SFTI-1 forms two anti-parallel β-strands that are connected centrally by a disulfide bridge that splits the molecule into a binding loop containing the reactive site and a secondary loop.[3] The primary structure of SFTI-1 is shown in Figure 1.

Owing to its small size and the well-defined structure, SFTI-1 has been chosen by several research teams to be a lead structure in the design of new inhibitors of trypsin, chymotrypsin and also physiologically important serine proteinases including human elastase,[4,5] cathepsin G,[6,7] matriptase,[8–10] β-tryptase,[11] proteinase K[12] and thrombin.[10] SFTI-1 has also become an excellent model peptide to determine the role of the Bowman-Birk inhibitor P'_2,[13] P'_3,[14] P'_4,[15] positions in the interactions with proteinases. The results summarizing the influence of other inhibitor positions of the binding loop were published in an excellent review.[4]

One of the first questions addressed to SFTI-1 was the role of its cyclic fragments (disulfide bridge and head-to-tail cyclisation) played in maintaining its rigid structure and how it influenced its high inhibitory activity. In our first paper on SFTI-1,[16] we were able to demonstrate that elimination of one cycle resulted in extremely potent inhibitors. The monocyclic SFTI-1 with a single disulfide bridge inhibited bovine β-trypsin with the same strength (as shown by the determined association constant, K_a) as did the wild inhibitor, whereas a head-to-tail cyclised analogue was only 2.5-fold weaker. Our results were subsequently confirmed by Korsinczky et al.[17] Based on conformational analysis of monocyclic SFTI-1 analogues, they proved that the disulfide bridge was essential for maintaining the 3D structure of the peptides. Also the solution structure of the head-to-tail cyclised analogue was similar (but less rigid) to that of the native SFTI-1. The authors also have found that the presence of the disulfide bridge in the sequence of SFTI-1 increased its proteolytic stability.[17] This finding is again in a good agreement with our previous[16] and more recent results describing kinetic studies of peptomeric analogues of this inhibitor.[18,19]

To obtain more proteolytic resistant SFTI-1 analogues, there have been made several attempts of modification of the disulfide bridge. For instance, Jiang et al.[8] synthesized SFTI-1 analogues with

Gly-Arg-Cys-Thr-Lys^5-Ser^6-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp

Figure 1. Primary structure of trypsin inhibitor SFTI-1.

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doi:10.1016/j.bmc.2010.10.014

disulfide bridge substituted by ethylene and olefin bridge. The first analogue inhibited matriptase 2.5-fold weaker than did SFTI-1. Again, the inhibitory activity of the olefin-bridged peptide was 25-fold weaker. Interestingly enough, both analogues were metabolically stable. Among the SFTI-1 analogues reported by Li et al.,[10] the most potent matriptase inhibitor appeared to be a methylenedithioether-bridged peptide. Its inhibition constant, K_i, with human matriptase almost matched that of the wild SFTI-1, and the analogue was more redox-stable than the parent compound. This led the authors to the conclusion that the peptide would be a promising drug candidate. Also the analogue with disulfide bridge formed by homo-L-cysteine (Hcy) introduced in position 3 displayed a high matriptase inhibitory activity. In our recent work[20] we described a series of SFTI-1 analogues in which the disulfide bridge was replaced by different-sized carbonyl bridges formed by side-chain amino groups of Lys, Orn, Dab or Dap located at the positions 3 and/or 11. All the analogues appeared to be strong trypsin inhibitors, proving that this fragment was well tolerate in the structure of the inhibitor. Our previous results[21] have shown that introduction of three mixed Cys–Pen (L-penicillamine) or Cys–Hcy disulfide bridges into the trypsin inhibitor EETI-II resulted in retaining inhibitory activity.

Bearing in mind the aforementioned results, we decided to synthesize a series of SFTI-1 monocyclic analogues in which the disulfide bridge was formed by combination of Cys, Hcy, Pen and Nhcy (*N*-sulfanylethylglycine) introduced at the positions 3 and/or 11, originally occupied by the Cys residues. Nhcy is an N-substituted glycine (the amide proton is substituted by a sulfanylethyl moiety) (see Fig. 2). Such amino acid mimetics are called peptoids. *N*-sulfanylethylglycine resembles homocysteine and therefore it was labelled Nhcy. We have already proven that peptoid monomers, resembling proteiongenic Lys and Phe, introduced in the inhibitor's substrate specificity P_1 position of serine proteinase inhibitors are recognized well by the enzymes.[18] Also substitution of amino acids located outside the inhibitor's reactive site by such derivatives preserved inhibitory activity of the modified serine proteinase inhibitors.[19] This was the reason why in synthesized analogues at the position P_1, in addition to Lys or Phe, we introduced peptoid monomers either *N*-(4-aminobutyl)glycine (Nlys) or *N*-benzylglycine (Nphe) that mimic those proteinogenic amino acids. Our purpose was to design SFTI-1 analogues with affinity to trypsin or chymotrypsin.

Taking into the account all the above mentioned results we synthesized the following series of monocyclic SFTI-1 analogues: [Nlys^5,Pen^3]SFTI-1 (**1**), [Nlys^5,Pen^{11}]SFTI-1 (**2**), [Nlys^5,Pen^{3,11}]SFTI-1 (**3**), [Nphe^5,Pen^3]SFTI-1 (**4**), [Nphe^5,Pen^{11}]SFTI-1 (**5**), [Nphe^5,Pen^{3,11}]SFTI-1 (**6**), [Hcy^{3,11}]SFTI-1 (**7**), [Phe^5,Hcy^{3,11}]SFTI-1 (**8**), [Nhcy^{3,11}]SFTI-1 (**9**), [Nlys^5,Nhcy^{3,11}]SFTI-1 (**10**), [Phe^5,Nhcy^{3,11}]SFTI-1 (**11**), [Nphe^5,Nhcy^{3,11}]SFTI-1 (**12**), [Phe^5,Nhcy^3]SFTI-1 (**13**), and [Phe^5, Nhcy^{11}]SFTI-1 (**14**).

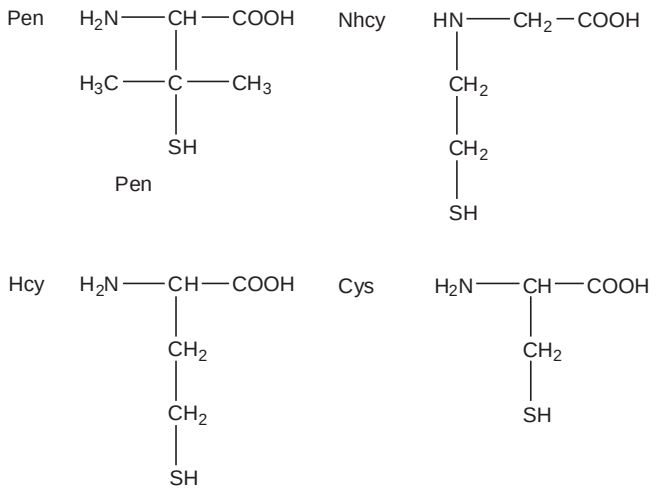

Figure 2. Chemical structure of a residues which were introduced in position 3 and/or 11.

2. Materials and methods

2.1. Peptide synthesis

All the peptides were synthesized by the solid-phase method using Fmoc chemistry. The following amino acid derivatives were used: Fmoc-Gly, Fmoc-Arg(Pbf), Fmoc-Cys(Trt), Fmoc-Hcy(Trt), Fmoc-Pen(Trt), Fmoc-Thr(*t*Bu), Fmoc-Lys(Boc), Fmoc-Ser(*t*Bu), Fmoc-Ile, Fmoc-Pro, Fmoc-Phe and Fmoc-Asp(O*t*Bu). The *C*-terminal amino acid residue, Fmoc-Asp(O*t*Bu), was attached to the 2-chlorotrityl chloride resin (substitution of Cl 1.46 meq/g; Calbiochem-Novabiochem AG, Switzerland) in the presence of an equimolar amount of DIPEA based on the amino acid in anhydrous condition, in DCM solutions. Peptide chains were elongated in the consecutive cycles of deprotection and coupling. Deprotection was performed with 20% piperidine in DMF/NMP (1:1, v/v) with addition of 1% Triton X-100, whereas the chain elongation was achieved with standard DIC/HOBt chemistry; 3 equiv of the protected amino acid derivatives were used. Peptoid monomers [Nphe, Nlys(Boc) and Nhcy(Trt)] were introduced into the peptide chain by the submonomeric approach.[22] In the first step, bromoacetic acid (5 equiv) was attached to the peptidyl-resin using DIC/HOBt methodology, followed by nucleophilic replacement of bromine with primary amines: benzylamine (8 equiv), *N*-1-Boc-1,4-diaminobutane × HCl (4 equiv) and *S*-tritylcysteamine (4 equiv) After completing the syntheses, the peptides (analogues **1**–**14**) were cleaved from the resin simultaneously with the side chain protecting groups in a one-step procedure, using a TFA/phenol/triisopropylsilane/H_2O (88:5:2:5, v/v/v/v) mixture.[23] In the last step, the disulfide bridge formation was performed using a 0.1 M methanolic iodine solution and the procedure described elsewhere.[24] The crude peptides were purified by HPLC on a Beckman Gold System (Beckman, USA) using an RP Kromasil-100, C_8, 5 μm column (8 × 250 mm) (Knauer, Germany). The solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). Either isocratic conditions or linear gradient were applied (flow rate 3.0 mL/min, monitored at 226 nm). The purity of the synthesized peptides was checked on a Pro Star system (Varian, Australia) equipped with a Kromasil 100 C_8 column (8 × 250 mm) (Knauer, Germany) and a UV–vis detector. The solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). A linear gradient from 10% to 90% B for 40 min with a flow rate of 1 ml/min was employed and monitored at 226 nm. The mass spectrometry analysis was carried out on a MALDI MS (a Biflex III MALDI-TOF spectrometer, Bruker Daltonics, Germany) using an α-cyano-4-hydroxycinnamic acid matrix.

Additionally selected compounds (**3** and **14**) have been characterized using ^{1}H NMR using multiple experiments including two dimensional analysis (2D TOCSY and NOESY) using NMR spectrometer Mercury VX 400 MHz (Varian Inc., USA). The results of the NMR studies are included in Supplementary data.

2.2. Determination of the association equilibrium constants

The detailed procedure of determination of association equilibrium constants are provided in Supplementary data. Briefly, the bovine β-trypsin (Sigma Chem. Co. USA) concentration was determined by spectrophotometric titration with 4-nitrophenyl-4′-guanidinobenzoate (NPGB) at an enzyme concentration oscillating around 10^{-6} M.[25] A standardized trypsin solution was used to

titrate ovomucoid from turkey egg whites, which in turn served to determine the solution concentration of the bovine α-chymotrypsin (Sigma Chem. Co., USA). The concentrations of the SFTI-1 analogues were determined by titration of their stock solutions with standardized bovine β-trypsin and bovine α-chymotrypsin, with N^{α}-benzoyl-DL-arginine-4-nitroanilide (BAPNA) and Suc-Ala-Ala-Pro-Leu-4-nitroanilide as substrates, respectively. The association constants (K_a) were measured by the method developed in the laboratory of M. Laskowski, Jr.[26,27] Enzyme–inhibitor interactions were determined in the 0.1 M Tris–HCl (pH 8.3) buffer containing 20 mM $CaCl_2$ and 0.005% Triton X-100, at room temperature. Increasing amounts of the inhibitor, varying from 0 to $2E_0$ (E_0—the total enzyme concentration), were added to the fixed amount of the enzyme. After a 3-h incubation, the residual enzyme activity was measured on a Cary 3E spectrophotometer (Varian, Australia) using a turnover substrate. The measurements were carried out at total enzyme concentrations over the ranges 5.3 and 8.0 nM for trypsin and chymotrypsin, respectively. The residual enzyme activity was measured with Ac-Ile-Arg-Asp-Asn-Lys-ANB-NH_2[28] and Suc-Ala-Ala-Pro-Leu-4-nitroanilide as chromogenic substrates for trypsin and chymotrypsin inhibitors, respectively. In all cases, the total substrate concentration was kept below 0.1 K_M. The experimental points were analysed by plotting the residual enzyme concentration [E] versus the total inhibitor concentration [I_0]. The experimental data were fitted to the theoretical values using the GraFit software package.[29]

2.3. Determination of proteolytic stability

The SFTI-1 analogues with modified disulfide bridges at concentration around 10^{-4} M were incubated in a 100 mM Tris–HCl buffer (pH 8.3) containing 20 mM $CaCl_2$ and 0.005% Triton X-100 using catalytic amounts of bovine β-trypsin or bovine α-chymotrypsin (1 mol %).[30] The incubation was carried out at room temperature and samples of the mixture were taken out periodically and submitted to RP-HPLC analysis. The analysis were performed on a Pro Star system (Varian, Australia) equipped with a Kromasil 100 C_8 column (8 × 250 mm) (Knauer, Germany) and a UV–vis detector. The solvent system was 0.1% TFA (A) and 80% acetonitrile in

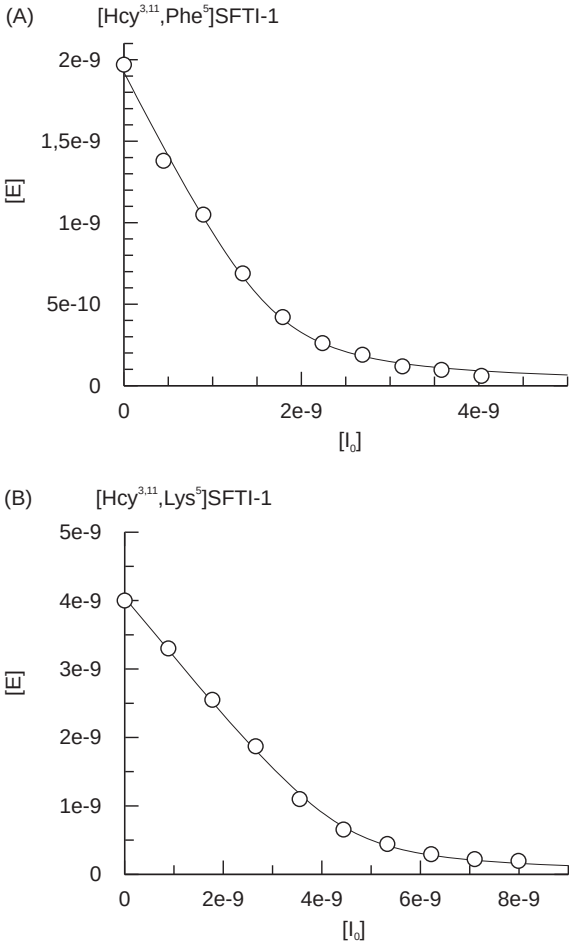

Figure 3. Inhibition curves of two most active inhibitors: (A) of trypsin—analogue **7**; (B) of chymotrypsin—analogue **8**. The equation used for calculation the association constant (K_a) is given below: $[E] = \frac{1}{2} \times \left([E_0] - F \times [I_0] - 10^{-logK_a} + \sqrt{([E_0] + F \times [I_0] + 10^{-logK_a})^2 - 4 \times F \times [I_0] \times [E_0]}\right)$.

Table 1
Physicochemical properties and association equilibrium constants (K_a) with bovine β-trypsin and bovine α-chymotrypsin of SFTI-1 analogues modified in disulfide bridge

Analogue[a]	MW calcd (found)[b]	RT [c] (min)	K_a (M^{-1})	
			Trypsin	Chymotrypsin
SFTI-1 wild[16,31]	1513.8 (1513.4)	16.7	$(1.1 \pm 0.2) \times 10^{10}$	$(5.2 \pm 1.6) \times 10^{6}$
SFTI-1[16]	1531.8 (1531.8)	13.7	$(9.9 \pm 1.1) \times 10^{9}$	$(4.9 \pm 1.4) \times 10^{6}$
[Phe^5]SFTI-1[6]	1550.2 (1550.8)	20.6		$(2.0 \pm 0.2) \times 10^{9}$
[Nlys^5]SFTI-1[18]	1531.8 (1531.5)	15.5	$(1.4 \pm 0.7) \times 10^{8}$	
[Nphe^5]SFTI-1[18]	1550.8 (1550.5)	20.1		$(3.9 \pm 0.3) \times 10^{8}$
[Nlys^5,Pen^3]SFTI-1 (**1**)	1559.9 (1559.9)	18.66	$(8.37 \pm 0.16) \times 10^{3}$	
[Nlys^5,Pen^{11}]SFTI-1 (**2**)	1559.9 (1559.6)	18.94	$(3.64 \pm 0.35) \times 10^{8}$	
[Nlys^5,Pen^{3,11}]SFTI-1 (**3**)	1587.9 (1587.8)	19.64	$(1.04 \pm 0.03) \times 10^{4}$	
[Nphe^5,Pen^3]SFTI-1 (**4**)	1578.9 (1578.5)	23.12		$(1.15 \pm 0.03) \times 10^{5}$
[Nphe^5,Pen^{11}]SFTI-1 (**5**)	1578.9 (1579.0)	23.57		$(1.40 \pm 0.13) \times 10^{8}$
[Nphe^5,Pen^{3,11}]SFTI-1 (**6**)	1606.9 (1606.4)	24.64		$(3.11 \pm 0.09) \times 10^{5}$
[Hcy^{3,11}]SFTI-1 (**7**)	1559.9 (1559.6)	20.53	$(7.24 \pm 1.18) \times 10^{9}$	
[Phe^5,Hcy^{3,11}]SFTI-1 (**8**)	1578.8 (1578.5)	24.07		$(7.56 \pm 1.79) \times 10^{9}$
[Nhcy^{3,11}]SFTI-1 (**9**)	1559.8 (1559.7)	18.72	NA	
[Nlys^5,Nhcy^{3,11}]SFTI-1 (**10**)	1559.8 (1559.6)	18.35	NA	
[Phe^5,Nhcy^{3,11}]SFTI-1 (**11**)	1578.8 (1580.2)	26.54		NA
[Nphe^5,Nhcy^{3,11}]SFTI-1 (**12**)	1578.8 (1578.9)	26.01		NA
[Phe^5,Nhcy^3]SFTI-1 (**13**)	1565.8 (1564.1)	23.97		NA
[Phe^5,Nhcy^{11}]SFTI-1 (**14**)	1565.8 (1565.5)	23.09		$(4.79 \pm 0.54) \times 10^{8}$

[a] With the exception of wild SFTI-1, the remaining analogues are monocyclic with disulfide bridge only.
[b] Molecular masses of the peptides were determined on a Bruker Biflex III MALDI-TOF spectrometer (Bruker, Germany). The average values are given.
[c] HPLC was performed as described in the Materials and methods section. The following linear gradients were applied: 10–90% B in 40 min and 15–80% B in 20 min (for SFTI-1 wild and SFTI-1), RT retention time, NA not active.

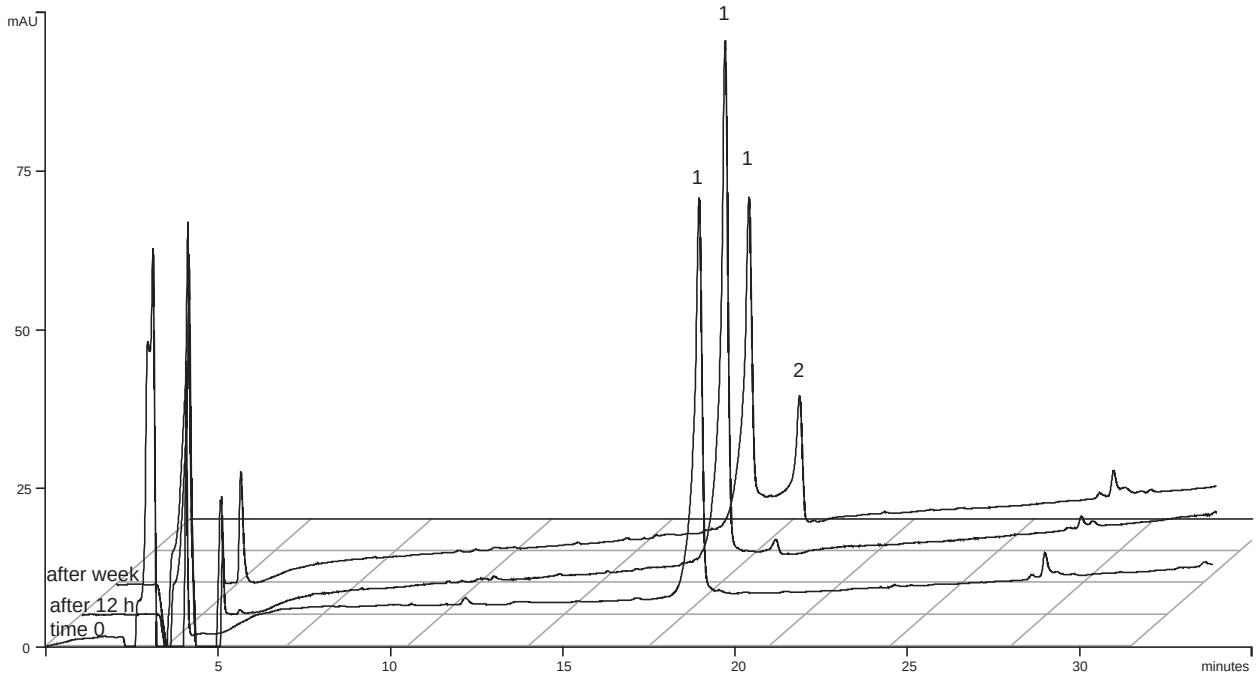

Figure 4. Proteolytic susceptibility of [Nlys[5],Pen[11]]SFTI-1—analogue **2**, in the presence of β-trypsin, at pH 8.3: peak 1—intact peptide, peak 2—fragment Cys-Thr-Nlys-Ser-Ile-Pro-Pro-Ile-Pen-Phe-Pro-Asp.

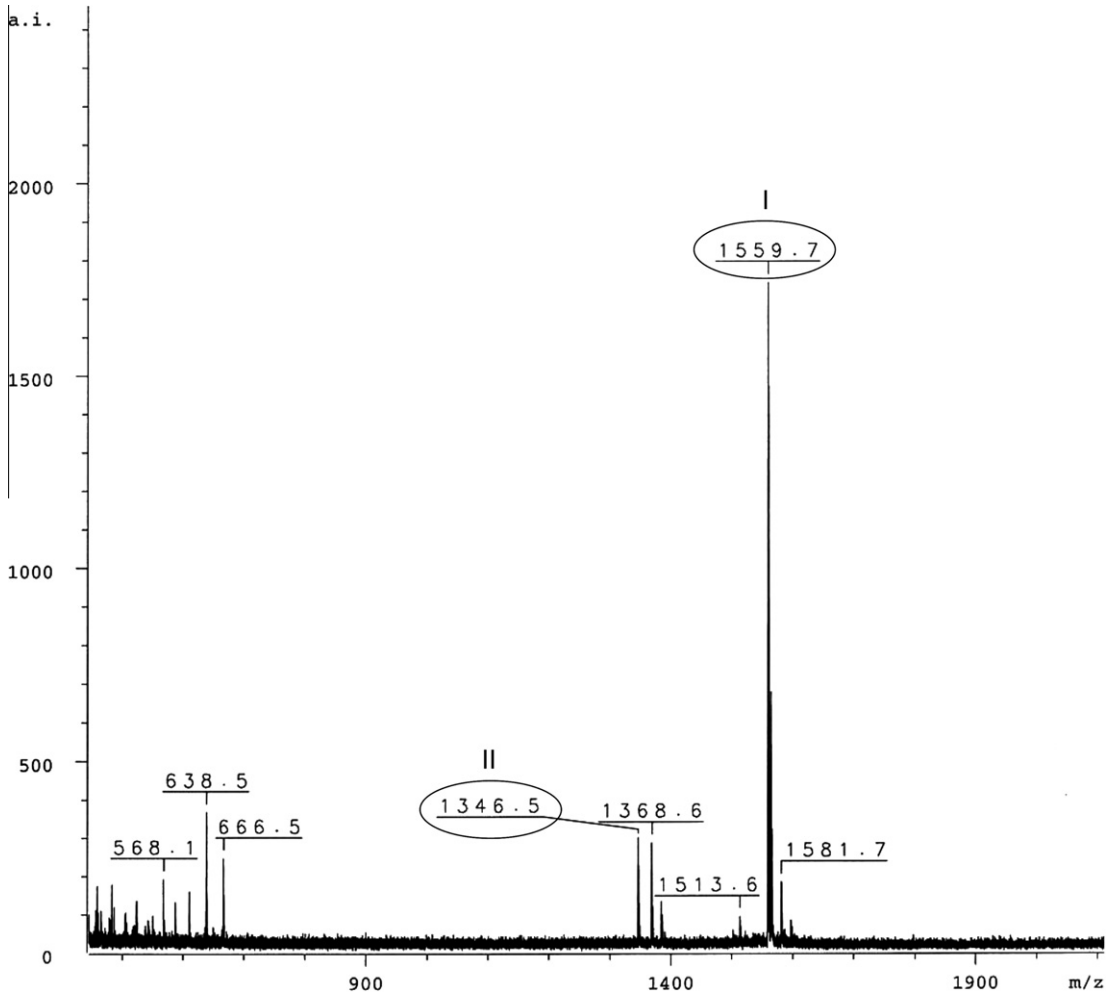

Figure 5. MS spectrum of peptide's fragments obtained after the incubation of analogue **2** in the presence of β-trypsin, at pH 8.3, incubation time 48 h: peak I—analogue **2** (peak 1 from 4.) peak II—fragment Cys-Thr-Nlys-Ser-Ile-Pro-Pro-Ile-Pen-Phe-Pro-Asp (peak 2 from Fig. 4).

A (B). Linear gradient was from 10% to 90% B for 40 min, flow rate 1 mL/min, monitored at 226 nm. The collected fractions of the peaks were analyzed by MALDI MS (Biflex III MALDI-TOF spectrometer, Bruker Daltonics, Germany) using the α-cyano-4-hydroxycinnamic acid matrix.

3. Results and discussion

The trypsin and chymotrypsin inhibitory activities, expressed as the K_a values with bovine β-trypsin or bovine α-chymotrypsin for the SFTI-1 analogues are summarized in Table 1. Inhibition curves of two most active inhibitors of trypsin (analogue **7**) and chymotrypsin (analogue **8**) are shown in Figure 3. The presented results clearly indicate that the role of amino acids located at the positions 3 and 11 forming the disulfide bridge in SFTI-1 is distinctly different. In the case of analogues modified by Pen and Nhcy, replacement of the Cys3 residue gave analogues with dramatically low inhibitory activity. Analogue **1** with the Pen residue at the position 3 displayed an over four orders of magnitude lower trypsin inhibitory activity as compared with that of [Nlys^5]SFTI-1 used as a reference compound. Additional substitution of Cys11 by Pen (analogue **3)** did not have significant impact on the inhibitory activity.

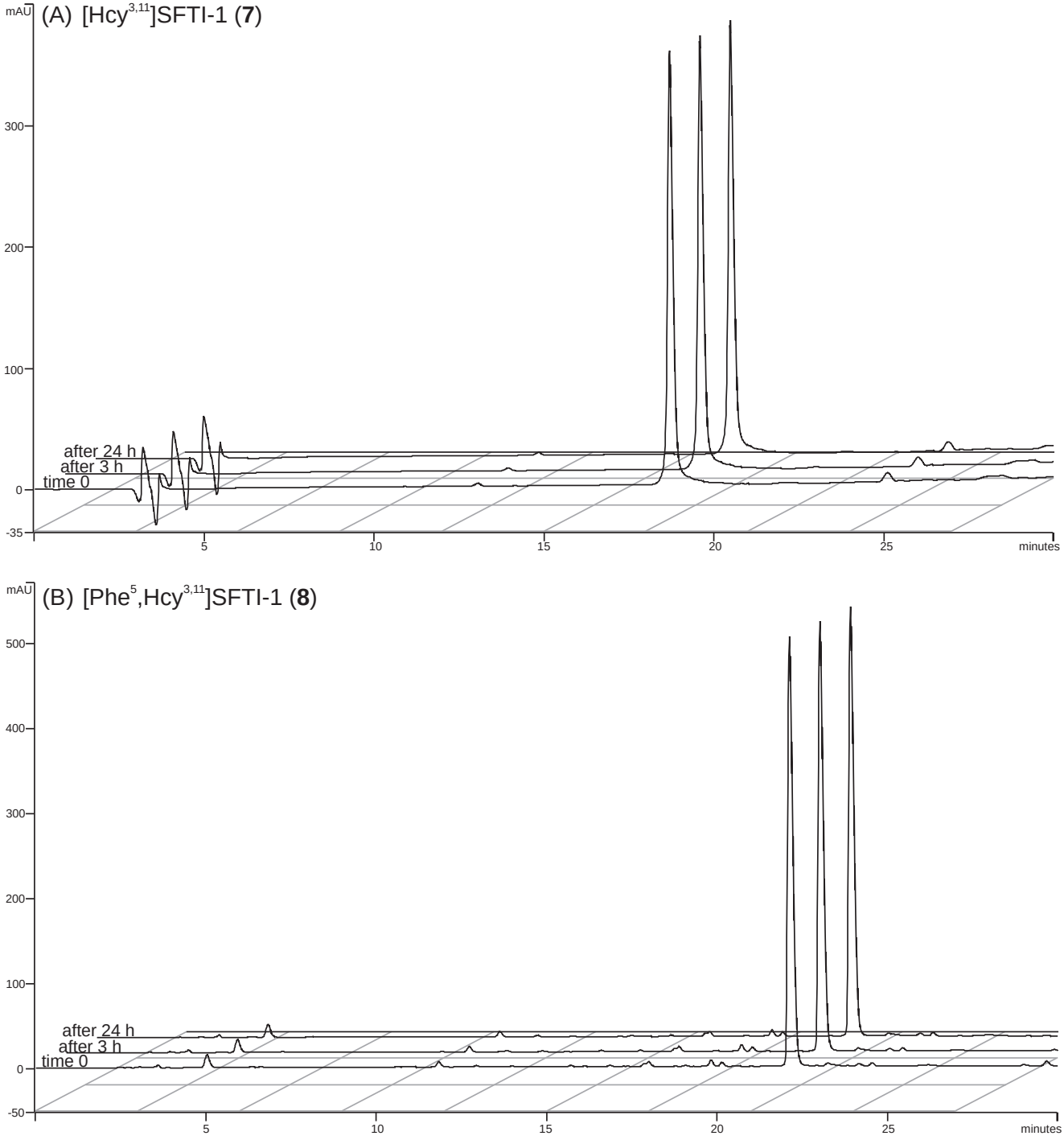

Figure 6. Proteolytic susceptibility of analogues: (A) [Hcys^{3,11}]SFTI-1 analogue **7**; (B) [Hcys^{3,11},Phe^5]SFTI-1 analogue **8**.

On the other hand, the K_a value of analogue **2** modified by Pen in the position 11 only, was even more than twice higher as compared with that of [Nlys^5]SFTI-1. Exactly the same relation was observed among analogues with Nphe in the inhibitor's P_1 position. Only analogue **5** with the Cys residue at the position 3 appeared to be a potent chymotrypsin inhibitor. Its K_a value with this enzyme is of the same order of magnitude as that for an analogue with the disulfide bridge formed by two Cys residues. The remaining two analogues, **4** and **6**, with Pen–Cys and Pen–Pen disulfide bridges respectively, were about three orders of magnitude less active.

Extension of the Cys3 and Cys11 side chains by one methylene group is well tolerated by both experimental enzymes. Analogues **7** and **8** with the Hcy–Hcy disulfide bridges are potent trypsin and chymotrypsin inhibitors, respectively. Analogue **7** preserved full activity of the monocyclic SFTI-1 whereas **8** was even several times more active than [Phe^5]SFTI-1.[6] Contrary to our previous results indicating that peptoid monomers are acceptable in the inhibitor's structures, displacement of the side chain from α-carbon of Hcy3 to nitrogen completely destroyed affinity of the modified analogues towards both experimental enzymes. All the analogues (**9**–**13**) with the Nhcy–Nhcy or mixed Nhcy–Cys disulfide bridges failed to inhibit the activity of trypsin and chymotrypsin. It is worth noting that the presence of reverse Cys–Nhcy disulfide bridge is fully accepted by chymotrypsin and the inhibitory activity of analogue **14** was practically the same as that previously determined for the analogue with such a cyclic fragment formed by two Cys residues.

In one of our recent works[18] we have shown that the disulfide bridge formed by two Cys residues significantly increased proteolytic stability of peptomeric SFTI-1 analogues. Under conditions described in the experimental section, all the active analogues appeared to be resistant to proteolysis. An example of proteolytic stability of [Nlys^5,Pen^{11}]SFTI-1 (**2**) is shown in Figure 3. As seen, a peak (with retention time of 18.9 min) corresponding to the intact peptide, is present at the beginning of incubation and after 48 h. As can also be seen in Figure 4, after prolonged incubation, a small peak (less than 3%) with retention time of 20.1 min appears. MALDI MS analysis of the fraction containing that peak revealed (see Fig. 5.) that it resulted from cleavage of the *N*-terminal Gly-Arg fragment from the inhibitor by bovine β-trypsin. This notwithstanding, it should be emphasized that the reactive $P_1 - P_1'$ site is fairly resistant to proteolysis under experimental conditions. The proteolytic stability of two most active analogues **7** and **8** is shown in Figure 6. As far as all the remaining active analogues (**1**, **3**, **4**, **5**, **6** and **14**) are concerned, even after a 48-h incubation with experimental enzyme, only peaks of intact peptides were present in the chromatograms (not shown).

Our results compatible with those published by Jiang et al.[8] and Li et al.[10] They obtained matriptase inhibitors by substitution of the disulfide bridge in SFTI-1 by olefin, ethylene, methylene dithioether or a mixed Hcy–Cys bridge. In the first two analogues the length of the bridge is the same as in SFTI-1, whereas in the remaining two inhibitors, the cyclic fragment was enlarged by a methylene group. We have shown that even the introduction of the $-CH_2-$ group to both Cys residues (analogues **7** and **8** with Hcy–Hcy disulfide bonds) is acceptable by both experimental enzymes and in the case of analogue **8** such a modification increases its association strength with chymotrypsin several times.

The observed dramatically lower inhibitory activity of the analogues with Pen and Nhcy at the position 3 might be associated either with a steric hindrance caused by two methyl groups attached to β-carbon of Pen or with elimination of hydrogen bond(s) (analogues with Nhcy) crucial for enzyme–inhibitor interaction. Cys 3 occupies inhibitor's P_3 and is part of its primary contact region with the enzyme. As shown by Luckett et al.,[1] NH and the carbonyl group of that amino acid residue come into contact with equivalent groups of trypsin Gly216. These contacts seem to be crucial for enzyme–inhibitor interactions. To verify this hypothesis, we decided to analyze complexes between trypsin and SFTI-1 analogues modified at the positions 3 or 11 by Pen and Nhcy. We performed a molecular dynamics simulation (see Supplementary data) on modified 3D structures of SFTI-1—bovine β-trypsin by replacing inhibitor's Cys3 and/or Cys11 by (Pen or Hcy) in the crystal structure of a complex available in the Protein Data Bank (PDB code: 1SFI). Unfortunately, the calculated energy of association between the enzyme and SFTI-1 analogues did not correlate with experimental K_a values (see Supplementary data).

Acknowledgement

This work was supported by Ministry of Science and Higher Education (Grant No. 2889/H03/2008/34).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.10.014.

References and notes

1. Luckett, S.; Santiago Garcia, R.; Barker, J. J.; Konarev, A. V.; Shewry, P. R.; Clarke, A. R.; Brady, R. L. *J. Mol. Biol.* **1999**, *290*, 525.
2. Schechter, I.; Berger, A. *Biochem. Bophys. Res. Commun.* **1967**, *27*, 157.
3. Korsinczky, M. L. J.; Schirra, H. J.; Craik, D. J. *Curr. Protein Pept. Sci.* **2004**, *5*, 351.
4. McBride, J. D.; Wtson, E. M.; Brauer, A. M.; Leatherbarrow, R. J. *Biopolymers* **2002**, *66*, 79.
5. Zabłotna, E.; Kret, A.; Jaśkiewicz, A.; Olma, A.; Leplawy, M. T.; Rolka, K. *Biochem. Biophys. Res. Commun.* **2006**, *340*, 823.
6. Łęgowska, A.; Dębowski, D.; Lesner, A.; Wysocka, M.; Rolka, K. *Bioorg. Med. Chem.* **2009**, *17*, 3302.
7. Łęgowska, A.; Dębowski, D.; Lesner, A.; Wysocka, M.; Rolka, K. *Mol. Divers.* **2010**, *14*, 51.
8. Jiang, S. J.; Li, P.; Lee, S.-L.; Lin, C.-Y.; Long, Y.-Q.; Johnson, M. D.; Dickson, R. B.; Roller, P. P. *Org. Lett.* **2007**, *9*, 9.
9. Long, Y.-Q.; Lee, S.-L.; Lin, C.-Y.; Enyedy, I. J.; Wang, S.; Li, P.; Dickson, R. B.; Roller, P. P. *Bioorg. Med. Chem.* **2001**, *11*, 2515.
10. Li, P.; Jiang, S.; Lee, S.-L.; Lin, C.-Y.; Johnson, M. D.; Dickson, R. B.; Micjejda, C. J.; Roller, P. P. *J. Med. Chem.* **2007**, *50*, 5976.
11. Scarpi, D.; McBridge, J. D.; Leatherbarrow, R. J. *Bioorg. Med. Chem.* **2004**, *12*, 6045.
12. Hilpert, K.; Hansen, G.; Wessner, H.; Volkmer-Engert, R.; Höhne, W. *J. Biochem.* **2005**, *138*, 383.
13. Gariani, T.; McBridge, J. D.; Leatherbarrow, R. J. *Biochim. Biophys. Acta* **1999**, *1431*, 232.
14. Daly, N. L.; Chen, Y.-K.; Foley, F. M.; Bansal, P. S.; Bharathi, R.; Clark, R. J.; Sommerhoff, C. P.; Craik, D. J. *J. Biol. Chem.* **2006**, *281*, 23668.
15. Brauer, A. B. E.; Domingo, G. J.; Cooke, R. M.; Matthews, S. J.; Leatherbarrow, R. J. *Biochemistry* **2002**, *41*, 10608.
16. Zabłotna, E.; Kaźmierczak, K.; Jaśkiewicz, A.; Stawikowski, M.; Kupryszewski, G.; Rolka, K. *Biochem. Biophys. Res. Commun.* **2002**, *292*, 855.
17. Korsinczky, M. L. J.; Clark, R. J.; Craik, D. J. *Biochemistry* **2005**, *44*, 1145.
18. Stawikowski, M.; Stawikowska, R.; Jaśkiewicz, A.; Zabłotna, E.; Rolka, K. *ChemBioChem* **2005**, *6*, 1057.
19. Łęgowska, A.; Bulak, E.; Wysocka, M.; Jaśkiewicz, A.; Lesner, A.; Dębowski, D.; Rolka, K. *Bioorg. Med. Chem.* **2008**, *16*, 5644.
20. Łęgowska, A.; Bulak, E.; Jaśkiewicz, A.; Małuch, I.; Sieracki, M.; Wysocka, M.; Lesner, A.; Rolka, K. *Prot. Pept. Lett.* accepted 10.05.2010.
21. Frąszczak, P.; Kaźmierczak, K.; Stawikowski, M.; Jaśkiewicz, A.; Kupryszewski, G.; Rolka, K. *Polish J. Chem.* **2002**, *76*, 1441.
22. Simon, R. J.; Kania, R. S.; Zuckermann, R. N.; Huebner, V. D.; Jewell, D. A.; Banville, S.; Ng, S.; Wang, L.; Rosenberg, S.; Marlowe, C. K.; Spellmeyer, D. C.; Tan, R.; Frankel, A. D.; Santi, D. V.; Cohen, F. E.; Bartlett, P. A. *PNAS* **1992**, *89*, 9367.
23. Sole, N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399.
24. Yang, Y.; Sweeney, W. V.; Schneider, K.; Chait, B. T.; Tam, J. P. *Protein Sci.* **1994**, *3*, 1267.
25. Chase, T.; Schaw, E. *Methods Enzymol.* **1970**, *19*, 20.
26. Empie, M. W.; Laskowski, M., Jr. *Biochemistry* **1982**, *21*, 2274.
27. Park, S. J. PhD thesis, Purdue University, (IN, USA), 1985.
28. Spichalska, B.; Lesner, A.; Wysocka, M.; Śledź, M.; Łęgowska, A.; Jaśkiewicz, A.; Miecznikowska, H.; Rolka, K. *J. Pep. Sci.* **2008**, *14*, 917.
29. Leatherbarrow, R. J. GraFit Version 5. Erithacus Software, Horley, 2005.
30. Jaulent, A. M.; Leatherbarrow, R. J. *Protein Eng. Des. Select.* **2004**, *17*, 681.
31. Zabłotna, E.; Kaźmierczak, K.; Jaśkiewicz, A.; Kupryszewski, G.; Rolka, K. *LIPS* **2002**, *9*, 131.