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

Anna Łęgowska *, Dawid Dębowski, Rafał Łukajtis, Magdalena Wysocka, Cezary Czaplewski, Adam Lesner, Krzysztof Rolka

Faculty of Chemistry, University of Gdansk, Sobieskiego 18, 80-952 Gdansk, Poland

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.

* Corresponding author.
E-mail address: legowska@chemik.chem.univ.gda.pl (A. Łęgowska).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
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**).

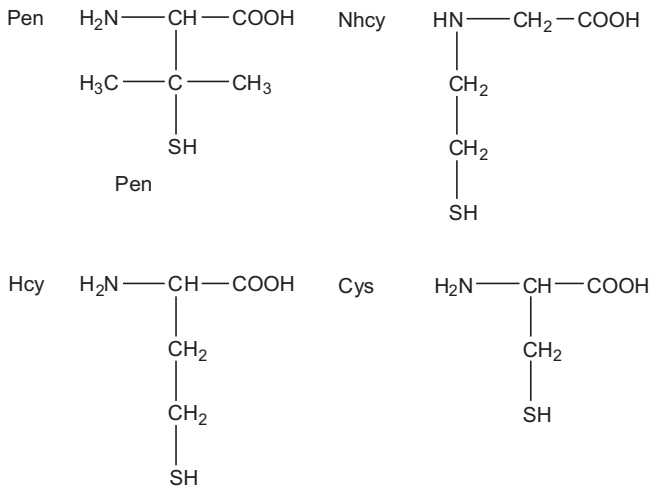

Figure 2. Chemical structure of a residues which were introduced in position 3 and/or 11.

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