Total chemical synthesis of bovine pancreatic trypsin inhibitor by native chemical ligation

Wuyuan Lu[a,*], Melissa A. Starovasnik[b], Stephen B.H. Kent[a]

[a] *Gryphon Sciences, 250 East Grand Avenue, Suite 90, South San Francisco, CA 94080, USA*
[b] *Department of Protein Engineering, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA*

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Abstract Bovine pancreatic trypsin inhibitor (BPTI) is an important model for the study of protein folding. Herein we describe a robust approach to the total chemical synthesis of BPTI using native chemical ligation of unprotected peptide segments in aqueous solution. After refolding and oxidative formation of disulfides, the target protein was purified by affinity chromatography. The synthetic BPTI was characterized by mass spectrometry, inhibition assay, thermal denaturation and 2D NMR spectroscopy, and was shown to be structurally and functionally identical to natural BPTI. The synthetic strategy presented in this paper has enabled us to establish rapid access to novel analogues of BPTI.

Key words: Bovine pancreatic trypsin inhibitor; Solid phase peptide synthesis; Native chemical ligation

1. Introduction

Bovine pancreatic trypsin inhibitor (BPTI) is a protein consisting of 58 amino acid residues with three disulfide bridges, and is arguably the most extensively studied small protein molecule. Studies on BPTI have covered almost all aspects of modern protein research, including: protein folding and stability; protein-protein recognition; protein (re)design; X-ray crystallography, and nuclear magnetic resonance (NMR) spectroscopy; calculation and molecular dynamics; biosynthesis and evolution; and, therapeutic treatment of diseases [1]. Not surprisingly, a strong interest in obtaining natural and/or unnatural analogues of BPTI has for years continued unabated, and the BPTI model system has stimulated methodological advances in protein production, from enzymatic and chemical semisynthesis to recombinant DNA-based expression technologies.

Semisynthesis [1], and particularly recombinant DNA-based expression [2,3], has played a dominant role in producing large quantities of BPTI analogues. Despite obvious potential advantages, total chemical synthesis has remained a rarely used approach to obtaining analogues of the protein. Even though several heroic total chemical syntheses of BPTI were reported in the 1970s [4–6], the homogeneity of some of these synthetic products would probably be called into question using today's standards for protein characterization. Recently, a successful synthesis of BPTI and two disulfide-lacking analogues using stepwise Fmoc SPPS was reported [7]. The synthetic wild type BPTI was found to be indistinguishable from the naturally occurring inhibitor by all criteria applied. The desire to understand the fundamentals of the BPTI model protein system means that total chemical synthesis and the systematic variation of covalent structure will play a more prominent role in the future.

In the past decade, solid phase peptide chemistry has witnessed tremendous technological advances in both synthesis [8,9] and analytical techniques [10]. Despite this progress, stepwise assembly of large polypeptide (>50 amino acid residues) still remains a challenging task due to the geometric increase in the numbers of resin-bound side products accumulated during a stepwise solid phase synthesis. This limitation of the size of synthetic polypeptide chains has been overcome by recently developed chemoselective ligation strategies [11], in particular, the powerful native chemical ligation technique [12]. The chemical ligation of unprotected peptide segments in aqueous solution is a robust and practical method for making synthetic polypeptide chains up to more than 100 amino acid residues [13]. Syntheses of a number of proteins of moderate size by a rapid single-step native chemical ligation have been reported, including interleukin-8 [12] and barnase [14], Abl SH3 [15], turkey ovomucoid third domain [16,17], human secretory phospholipase A2 [18] and a number of chemokines [19,20].

In this paper we describe the total chemical synthesis of wild type BPTI using native chemical ligation, in an effort to establish rapid and robust synthetic access to an expanded repertoire of analogues of the BPTI protein molecule.

2. Materials and methods

2.1. Materials

Boc-L-amino acids and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) were purchased from Novabiochem; Boc-Ala-OCH_2-Pam-resin and *N*,*N*-diisopropylethylamine (DIEA) were obtained from Applied Biosystems. Aminomethyl-resin was prepared by Dr. Michael Carrasco according to published procedures [21]. Acetonitrile was purchased from Fisher Scientific, trifluoroacetic acid (TFA) from Halocarbon Products Co., and HF from Matheson Gas Products. Bromoacetic acid and thiophenol were purchased from Aldrich Chemical Co. Bovine α-chymotrypsin, β-trypsin and natural BPTI purified from bovine pancreas were purchased from Worthington Biochemical Co. All chromogenic substrates used in inhibitory activity assays were obtained from Bachem Bioscience Inc. Anhydrotrypsin was prepared according to [22] and was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the coupling procedures provided by the manufacturer.

Analytical reversed phase HPLC was performed on a Hewlett Packard Series 1050, using a Vydac C-18 column (5 μm, 4.6×150 mm); preparative reversed phase HPLC was carried out on a Waters Delta Prep 4000 system using a Vydac C-18 column (15–20 μm, 50×250 mm); solvent A for HPLC was water containing 0.1% TFA; solvent B was 90% acetonitrile containing 0.1% TFA. Mass spectrometry analysis was carried out on a PE Sciex API-III quadrupole electrospray ionization mass spectrometer (ESI-MS). The inhibitor-enzyme association equilibrium constants were measured on a Hewlett-Packard

*Corresponding author. Fax: (1) (650) 952-3055.
E-mail: wuyuan_lu@gryphonsci.com

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HP8450A spectrophotometer by a modified version of the Green and Work method [23,24]. The experiment was carried out at 21 ± 1°C at pH 8.3 in 0.1 M Tris-HCl buffer containing 0.02 M $CaCl_2$ and 0.005% Triton X-100. Thermal denaturation was performed on a Calorimetry Sciences Nano-DSC microcalorimeter at pH 3.0. ^{1}H NMR spectra were acquired on a Bruker AMX500 spectrometer at 27°C, pH 5.3.

The principles of native chemical ligation, in which two unprotected peptide segments form a native peptide bond by forming an intermediate thioester ligation product followed by a spontaneous S→N acyl rearrangement, have been described [12,14]. The experimental procedures briefly outlined below were largely adopted from the synthesis of turkey ovomucoid third domain, another serine proteinase inhibitor of similar size [16,17]. The design of the synthetic strategy is illustrated in Fig. 1.

2.2. Synthesis of BPTI(1–37)αCOSH

The thioacid peptide was manually synthesized on Boc-Gly-(thioester linker)-aminomethyl-resin [25], using the optimized in situ neutralization/HBTU activation protocol for Boc chemistry [9]. After the chain assembly was complete, the peptide was deprotected and cleaved by HF in the presence of 5% *p*-cresol at 0°C for 1 h. To obtain the corresponding thioester peptide needed in the ligation reaction, the crude thioacid peptide BPTI(1–37)αCOSH was allowed to react with bromoacetic acid for 30 min (1 mg/ml bromoacetic acid, 5 mg/ml peptide, in 0.1 M NaOAc, 6 M GuHCl, pH 4.0). The thioester product (1–37)αCOS-CH_2COOH was subsequently purified by preparative C18 reversed phase HPLC using a gradient of 30–50% B (yield 15%). The molecular weight of the thioester peptide was ascertained by ESI-MS (observed 4289.9 ± 0.5 Da; calculated 4290.9 Da, average isotope composition).

2.3. Synthesis of BPTI(38–58)

The C-terminal peptide segment was manually synthesized on Boc-Ala-OCH_2-Pam-resin using the same chain assembly chemistry as described above. After deprotection and cleavage in HF, the crude peptide was purified by preparative C18 reversed phase HPLC running a gradient of 15–30% B (yield 25%). The molecular weight of the peptide was found to be 2318.3 ± 0.6 Da, which was in good agreement with the calculated value 2318.7 Da (average isotope composition).

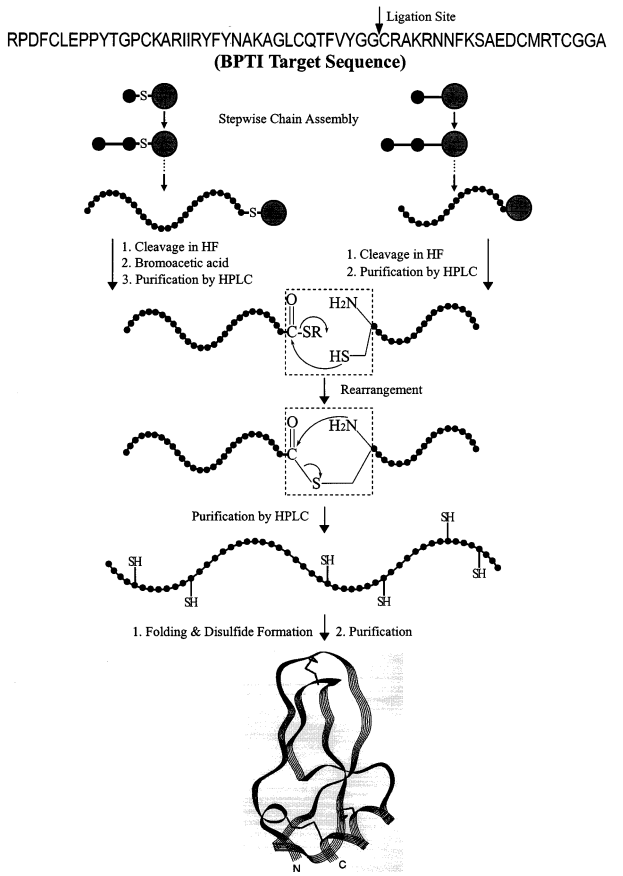

Fig. 1. Strategy for the synthesis of BPTI by native chemical ligation.

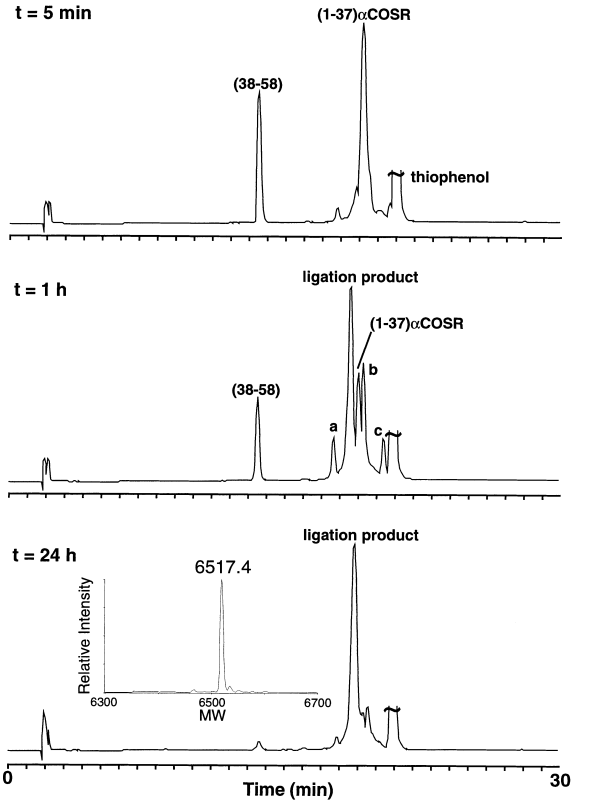

Fig. 2. Native chemical ligation of BPTI(1–37)αCOSR and BPTI(38–58). The ligation reaction was monitored by analytical C-18 RP-HPLC (0–67% B over 24 min) and ESI-MS. Peaks a, b and c represent reaction intermediates (for details see Section 2).

2.4. Native chemical ligation

BPTI(1–37)αCOS- CH_2COOH (56 mg, 13.1 μmol) and BPTI(38–58) (32 mg, 13.8 μmol) were dissolved in 8.8 ml of 0.1 M phosphate buffer, pH 7.5, containing 6 M GuHCl and 3% thiophenol. As shown in Fig. 2, the ligation reaction essentially went to completion in 24 h (most of the starting materials were actually converted to the product within 8 h). The ligated full-length BPTI polypeptide was purified by preparative C18 reversed phase HPLC (30–50%B) (yield 60%). The molecular weight of the ligation product, 6517.4 ± 0.6 Da, was in good accord to the calculated value of 6517.6 Da (average isotope composition). It is interesting to point out that there was a brief accumulation of intermediates (marked as peaks a, b, and c in Fig. 2) in the early stage of the ligation reaction. Further analysis by ESI-MS revealed that peaks a and b were probably two different cyclized thioester intermediate forms of (1–37)αCOS-CH_2COOH, and peak c was thiophenol ester peptide (1–37)αCOS-Ph.

2.5. Folding and purification

Protein folding was carried out by dissolving 52 mg (8.0 μmol) of reduced BPTI(1–58) at 1.2 mg/ml in 0.6 M Tris-HCl, 6 mM EDTA, 6 M GuHCl, pH 8.7, which was then diluted 6-fold with water. The folding solution was gently stirred in an open air container at room temperature overnight, and then was loaded onto an anhydrotrypsin-Sepharose affinity column. After washes with unbuffered salt solution, the bound fractions were eluted by lowering the pH to 2. The active protein collected from the affinity column was further purified by cation ion exchange SP-Sepharose chromatography at pH 4.8, followed by desalting and lyophilization (yield 45%).

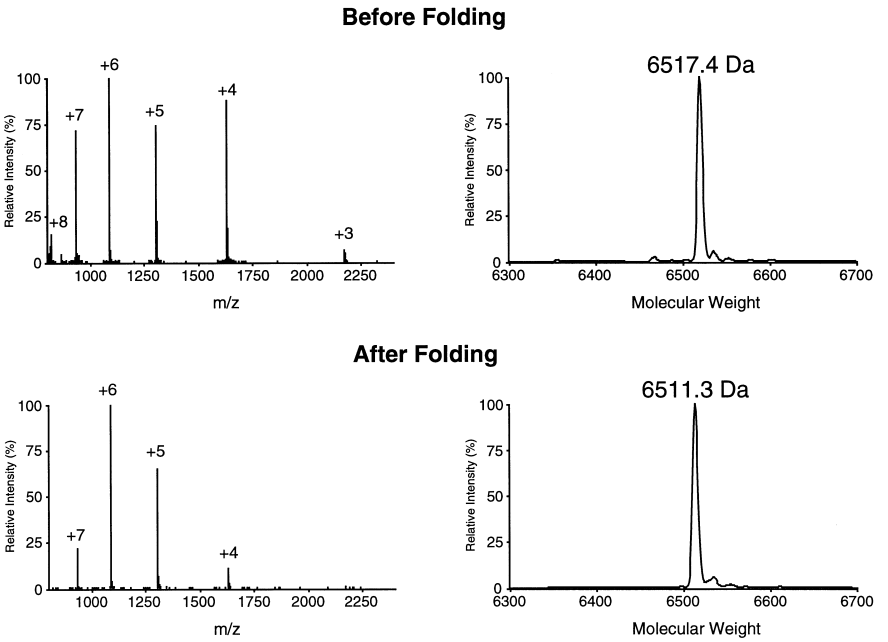

Fig. 3. ESI-MS analysis of BPTI before and after folding. The observed mass after folding is 6 Da lower than that of the fully reduced BPTI, as expected for the formation of three disulfides.

3. Results and discussion

3.1. Scale and yields

Peptide syntheses were carried out on 0.4 mmol of Boc-Ala-Pam resin and of Boc-Gly-(thioester linker)-aminomethyl resin. Yields of purified peptides were 25% for BPTI(38–58) (100 μmol) and 15% for BPTI(1–37)αCOS-CH_2COOH (60 μmol), respectively. Native chemical ligation was carried out on a 13 μmol scale, yielding 52 mg of full-length BPTI(1–58) (60%). Refolding and subsequent purification gave rise to 23 mg of active BPTI (45%). The overall yield of the synthesis was 4%, so that more than 100 mg of synthetic protein can be obtained from reaction and work-up of the two synthetic peptide segments.

The success of any synthesis, to a large extent, is dictated by the quality of the product. For this reason, we carefully characterized the synthetic BPTI.

3.2. Electrospray mass spectrometry

The synthetic product before and after folding was analyzed by ESI-MS (Fig. 3). The mass for the folded BPTI was found to be 6511.3 ± 0.7 Da (calculated 6511.6 Da, average isotope composition), whereas the molecular weight of the unfolded form was determined to be 6517.4 ± 0.6 Da (calculated 6517.6 Da, average isotope composition). The difference in the observed masses (6 units) for the oxidized and reduced BPTI indicate that three disulfide bridges, as expected, have formed during refolding. To demonstrate that the synthetic protein folded correctly, however, additional functional and structural characterizations were conducted as described below.

3.3. Inhibitory activity assay

BPTI is a strong inhibitor of bovine β-trypsin. Our synthetic BPTI inhibited bovine β-trypsin stoichiometrically (data not shown), indicating that it is a strong inhibitor of trypsin. Even though an estimated association equilibrium constant (K_a) of about 10^{14} M^{-1} has often been cited in the literature [1], few groups have actually been able to accurately measure a binding constant that high [26]. This is because the dissociation rate constant (k_{off}) for BPTI-trypsin complex is so low that an impractical amount of time will be needed in order for the system to reach equilibrium. For that reason, accurate measurement of the K_a value with trypsin was not attempted. Instead, K_a values for the synthetic BPTI and naturally occurring BPTI interacting with chymotrypsin were determined (Fig. 4). They are 8.4×10^7 M^{-1} and 7.7×10^7 M^{-1}, respectively, which are in good agreement with the values reported in the literature [1]. These results suggest that the synthetic BPTI is indeed functionally identical to the natural inhibitor.

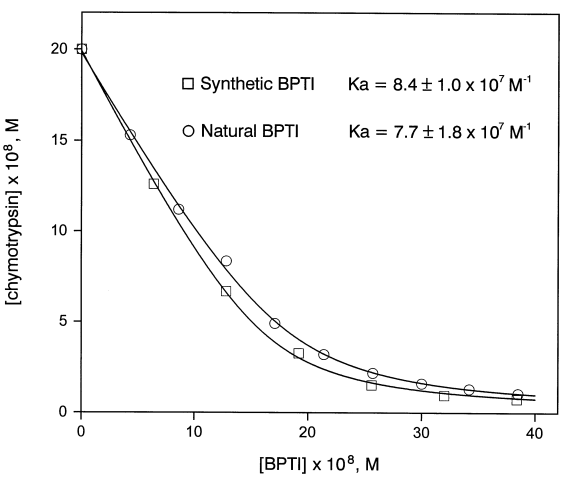

Fig. 4. Inhibition of bovine α-chymotrypsin by synthetic and natural BPTI.

3.4. Thermal denaturation

BPTI is one of the most stable small proteins ever reported [27]. To demonstrate the structural integrity of the synthetic BPTI, thermal denaturation was performed for both synthetic BPTI and natural BPTI on a differential scanning microcalorimeter at pH 3.0. As shown in Fig. 5, the two proteins have almost identical melting transition and melting temperatures, which strongly suggested that the synthetic BPTI was correctly folded.

3.5. ^1H NMR

In order to evaluate the three-dimensional structure of BPTI synthesized in this manner, 2D COSY, TOCSY, and NOESY spectra were acquired using standard methods. The spectra were entirely consistent with data reported previously for natural BPTI [28]. A portion of the TOCSY spectrum is shown in Fig. 6. This and other NMR data confirmed that the synthetic material was folded correctly, i.e. in the same conformation as native BPTI.

4. Conclusions

We have described a practical and robust synthetic access to BPTI by straightforward native chemical ligation, which allows for facile production of previously unobtainable analogues of this intensively studied model protein. In fact, using the synthetic strategy outlined in this paper, we have made and studied the properties of some novel topological analogues of the BPTI molecule (unpublished results). One obvious advantage of native chemical ligation is the ease with which a large number of analogues can be produced. Given the tremendous need for novel analogues of BPTI for the study of protein folding, protein stability, protein-protein recognition, and protein de novo design, etc., the establishment of robust synthetic access to BPTI is a timely development. Using the convenient total chemical synthesis described here, novel analogues not accessible by conventional (i.e. recombinant DNA-based) means in both side chains and the backbone of the BPTI protein molecule can be readily prepared. These analogues will provide the foundation for systematic dissection of the molecular basis of important aspects of the BPTI system, and will lead to improved understanding of protein structure/function relationships

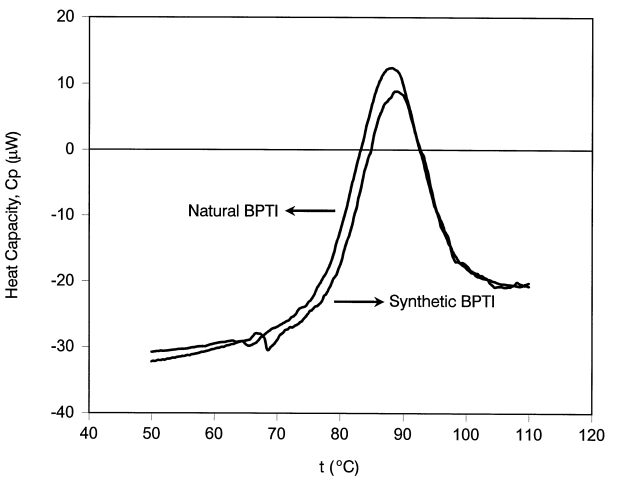

Fig. 5. Thermal denaturation by differential scanning calorimetry at pH 3.0.

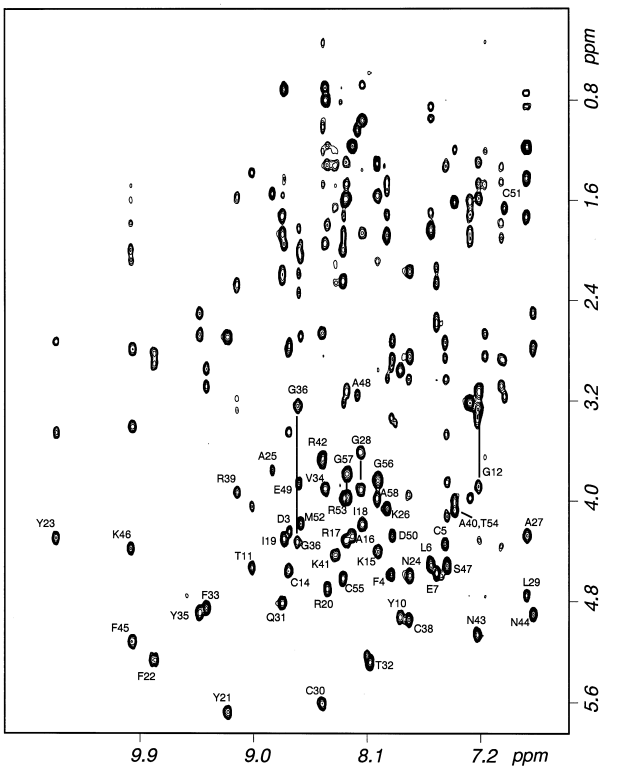

Fig. 6. Amide-to-aliphatic proton region of the 2D TOCSY spectrum acquired with an 85 ms mixing time. Assignments for cross peaks between amide and alpha protons are indicated.

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