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Facile and efficient chemical synthesis of APET×2, an ASIC-targeting toxin, via hydrazide-based native chemical ligation

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ABSTRACT

Acid-sensing ion channels (ASICs) targeting peptides APET×2, a minor constituent in the venom of the sea anemone *Anthopleura elegantissima*, was synthesized via hydrazide-based native chemical ligation with high yields. Synthetic APET×2 exhibited well-defined three-dimensional structure after an optimized folding process. Therefore, we present a simple and cost-efficient strategy for the chemical synthesis of ASIC3 inhibitor APET×2.

1. Introduction

As key pharmacological tools for studying ion channel function–structure relationships, animal toxins can target a variety of receptors and ion channels with high specificity and affinity.[1] These venom peptides were most used to determine the molecular targets for understanding their modes of action for basic science.[2] Moreover, many of these polypeptide toxins have undergone clinical trials and have been developed as venom-based drugs that inhibit neuronal channels involved in pain and other sensory transduction pathways.

Acid-sensing ion channels (ASICs) are voltage-independent proton-gated cation channels,[1a] which can be inhibited or potentiated by toxin proteins extracted from several animals, including PcT×1 (venoms of spider),[3] MitTx, Mambalgins (snakes)[4] and APET×2 (sea anemone).[5] Most of venom peptides were obtained by isolating from venoms of various origins, while the yield is typically low. The inability to isolate sufficient quantities of pure toxin has become a major bottleneck to further structural and functional studies. Recently, PcT×1 and Mambalgins were achieved by protein chemical synthesis with extremely high purity and efficiency.[6]

The sea anemone peptide APET×2 was the second ASIC-targeting peptide discovered in 2004.[5] This 42 amino acid protein, which displays 64% sequence identity with APET×1, can inhibit ASIC3 homomeric channels and ASIC3-containing heteromeric channels.[7] Very little work about the chemical synthesis of APET×2 was reported still Rash group work on this protein via native chemical ligation.[8] In spite of the improvements, efforts are still needed to overcome some constraints, for example, time-consuming steps for the synthesis of the thioester and its surrogates for ligation. Recently, the hydrazide based ligation developed by Liu group has been used for protein total and semi-synthesis and protein medications.[9] One of the main advantages is that peptide hydrazides can be easily prepared by using Fmoc solid-phase peptide synthesis.[10] Herein, we take the advantage of protein chemical synthesis with hydrazide-based native chemical ligation to obtain large quantities of the homogeneous and highly pure toxin. Moreover, the three-dimensional structure of the protein with rich disulfide bonds is also presented (Scheme 1).

2. Results and discussion

The sea anemone peptide APET×2 contains 42 amino acid residues, including three intramolecular disulfide bonds (C4-C37, C6-C30 and C20-C38). Firstly, we attempted to synthesize full-length

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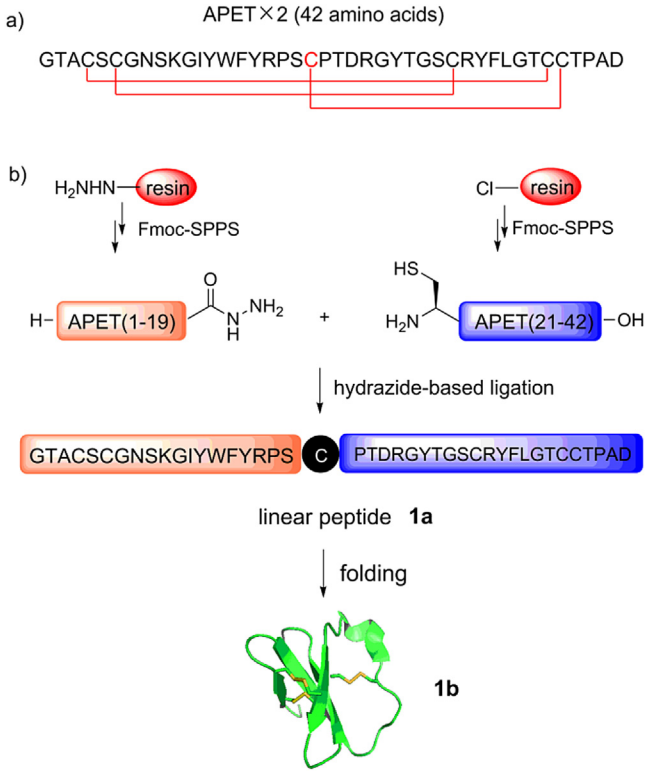

Scheme 1. The scheme for the chemical synthesis of APET×2.

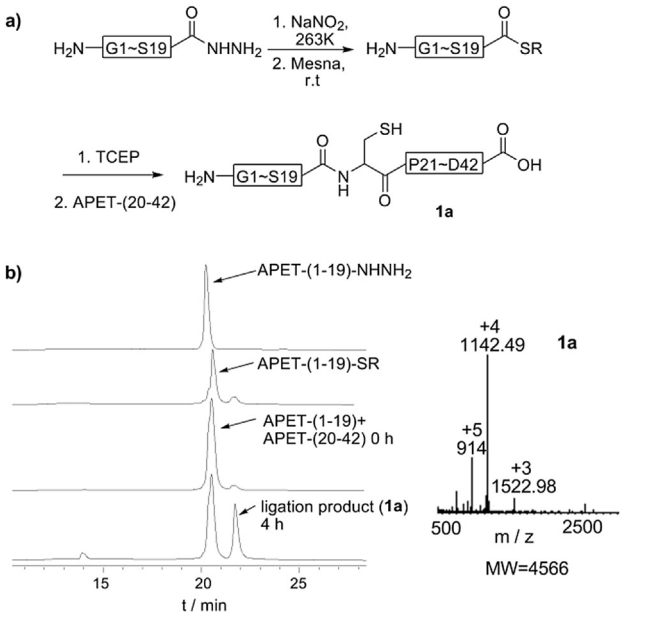

Fig. 1. a) Scheme of the ligation process; b) Analytical HPLC trace and ESI-MS for linear peptide APET×2 (obs: 4566 Da; calcd: 4567 Da).

APET×2 via standard Fmoc-based SPPS, while it failed even using optimized protocols. Then the protein was design to synthesize by two segment ligation by using hydrazide-based native chemical ligation (NCL). In detail, APET×2 was divided into two fragments, including APET-(G1-S19)-$NHNH_2$ (segment 1) and APET-(C20-D42)-COOH (segment 2). Two segment peptides can be obtained by Fmoc-SPPS in relative high yield (isolated yields 10% and 9%).[11] With peptide in hands, we first convert APET-(G1-S19)-$NHNH_2$ to APET-(G1-S19)-MesNa by treating with 5 mM $NaNO_2$ (pH 3.0) and 50 mM MesNa (pH 5.0) in reaction buffer (6 M Gu·HCl/0.1 M Na_2HPO_4). In this process, the proper pH (no high than 7) was important because APET-(G1-S19)-MesNa is easy to hydrolysis in alkaline conditions. Then, the APET-(C20-D42)-COOH (1.5 mM) was added into the reaction buffer (6 M Gu·HCl/0.1 M Na_2HPO_4) and the pH value was adjusted to 7.0. The reaction was monitored by using HPLC and the product peak was completed after 4 h. The linear APET×2 (**1a**) was further identified by ESI-MS and the isolated yield was 55% (Fig. 1).

To further obtain well-structure APET×2, we used in vitro folding of linear APET×2 to ensure correct formation of the three disulfide bridges. At the beginning, the method reported by Otaka group was used to refold linear APET×2,[12] while no obvious peak change was detected after 24 h stirring at room temperature. Then the temperature was decreased to 4 °C because low temperature should benefit for protein folding. However, there is no corrected folding product was obtained even we changed the folding buffer and the concentration of peptide similar as Kent group suggested.[13] In the process of experiment, we found that there was something insoluble product during long period stirring. Therefore, we attempted to add more Gu·HCl and further decrease the concentration of linear APET×2. Finally, the correct folded APET×2 was successfully obtained when the concentration of Gu·HCl (1.05 M) and linear APET×2 (0.025 mg/mL) were fully optimized (Table 1). It's worth noting that the pH value of folding buffer must decrease to 2–3 before lyophilized, otherwise the characters of folded APET×2 would restored to linear product. As shown in Fig. 2, we got well-folded APET×2 with relative high yield based on the optimized renaturation method after HPLC purification (isolated yield 46%).

Table 1
The reagents and the ambient temperature for optimizing the folding of linear APET×2

Gu·HCl/Na_2HPO_4 (M/M)	Tris·HCl (mM)	GSH/GSSG (mM/mM)	Tween 80 (v/v)	**1a** (mg/mL)	Isolated yield
0.5/0	100	8/1	0	0.5	N.R
0.5/0	100	8/1	0	0.01	N.R
1.05/0.0175	41.25	1.65/0.165	0.003%	0.25	N.R
1.05/0.0175	41.25	1.65/0.165	0.003%	0.025	46%

With well-folded compounds in hands, we next explored the three dimensional structure of synthetic APET×2 via circular dichroism (CD) spectrum. The folding of this synthetic peptide was found to occur automatically in PBS buffer (0.2 M Na_2HPO_4) at pH 7.5. The CD spectrum of **1b** in water indicated a negative minimum at 205 nm and a strong positive band at 229–240 nm (Fig. 3). These observations were in accord with the CD analysis of APET×2 as previously reported.[5]

To further determine the 3D structure of synthetic APET×2, we conducted solution NMR spectroscopy (including DQF-COSY, TOCSY and NOESY) (Fig. 4 and Table 2). For APET×2 peptide, a set of 300 structures were calculated using a total of 340 distance restrains comprising 173 intra-residue, 98 sequential, 27 medium-range and 42 long-range. 20 low overall energy and low constraint violation structures were selected for further analysis. The root-mean-square deviation (RMSD) from the structure ensembles of APET×2 is 0.828A for the backbone atoms and 1.185A for all the heavy atoms. The solution structures revealed that APET×2 adopted an ordered structure, which consists of a four-stranded β-sheet from a loop (15–27) and N and C terminal. Four anti-parallel β strands, including residues 3–6 (strand I), residues 9–14