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Synthesis and characterization of sapecin and sapecin B

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Abstract

Two insect defencins, sapecin and sapecin B, were chemically synthesized to confirm their structure and antibacterial activity and also to examine the possibility that these peptides bind to the same site on the large conductance calcium-activated potassium channel as charybdotoxin. Both synthetic peptides showed the same antibacterial activity as native sapecins, indicating that the synthetic peptides folded correctly in the chemical synthesis. Synthetic sapecins did not show an inhibitory effect on [^{125}I]charybdotoxin binding to rat brain synaptic membranes, suggesting that sapecin B recognizes a different binding site from that of charybdotoxin despite the similar structural motif.

Key words: Sapecin; Sapecin B; Charybdotoxin; Potassium channel

1. Introduction

Many insects produce antibacterial peptides in response to infection of bacteria or body injury. Sapecins (sapecin, sapecin B, and sapecin C) are a group of antibacterial peptides isolated from the culture medium of an embryonic cell line of *Sarcophaga peregrina* (flesh fly) [1,2]. These peptides have potent antibacterial activity against various Gram-positive bacteria. Sapecin is also found to stimulate embryonic cell proliferation [3]. Sapecins have three disulfide bonds in their structures (Fig. 1). Among sapecins, sapecin B is less similar to sapecin and sapecin C but shows significant similarity to charybdotoxin, an inhibitor of a large conductance calcium-activated potassium channel isolated from a scorpion venom [4,5]. In the present study, we synthesized two peptides having the same amino acid sequences as native sapecin and sapecin B in order to confirm their structure and antibacterial activity and also to examine the possibility that they bind to the same site on the potassium channel as charybdotoxin. Synthesis of these peptides in large quantities will also be useful for further study of the bacterial defence system as well as three-dimensional structure analysis.

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Abbreviations: Boc, *tert*-butyloxycarbonyl; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; ODS, octadesylsilane; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid; Trt, trityl.

2. Materials and methods

2.1. Materials

Fmoc-Cys(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Asp(OtBu)-OH, and other reagents used on the synthesizer were obtained from Applied Biosystems Japan (Chiba, Japan). Fmoc-Asn(Trt)- and Fmoc-Gln(Trt)-*p*-oxybenzyloxybenzyl alcohol resin were obtained from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). Other reagents were obtained from Peptide Institute (Osaka, Japan) or Kokusan Chemical Works Ltd. (Tokyo, Japan).

2.2. Peptide synthesis

Solid-phase peptide synthesis was conducted on an Applied Biosystems 431A peptide synthesizer. Amino acid analyses were performed on a Beckman System Gold amino acid analyzer after hydrolysis in 6 M hydrochloric acid at 110°C for 24 h and derivatization by 4-dimethylaminoazobenzene-4′-sulphonyl chloride. FAB-MS spectra were measured on a JEOL HX-110 mass spectrometer. Analytical HPLC was conducted on a Shimadzu LC-6A system with ODS column (4.6×250 mm). Preparative HPLC was performed with a Shimadzu LC-8A system with ODS column (20 × 250 mm).

Linear precursors of sapecin and sapecin B were assembled by solid-phase methodology of Fmoc chemistry starting from Fmoc-preloaded resin using a Trt group for the protection of SH groups of Cys residues. After TFA cleavage, crude linear peptides were extracted with 2 M AcOH and diluted to a peptide concentration of 0.02 mM. The solutions were adjusted to pH 7.8 with aqueous NH_4OH and stirred slowly at room temperature. The cyclization reaction was monitored by HPLC and the reaction mixture was loaded on to the column of carboxymethylcellulose CM-52 and eluted with 0.5 M ammonium acetate (pH 6.5). Crude linear peptides were purified by successive chromatographies with Sephadex G-50F and preparative HPLC with ODS columns. Structures and the purity of synthetic peptides were confirmed by analytical HPLC, amino acid analysis, and FAB-MS measurement.

2.3. Bioassay

The antibacterial activities of synthetic sapecin and sapecin B were estimated from the inhibitory effects on the growth of *Staphylococcus aureus* IFO 12732, as described previously [2]. The binding activities to

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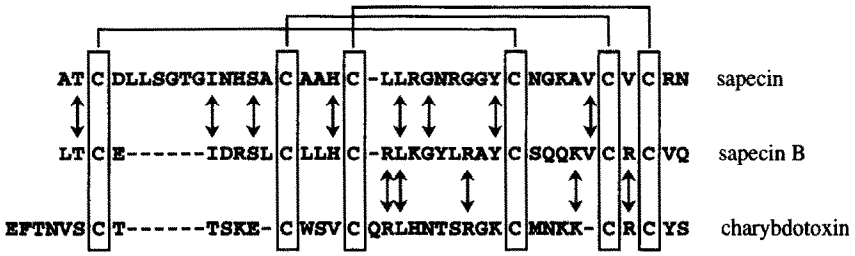

Fig. 1. Amino acid sequences of sapecin, sapecin B and charybdotoxin. Arrows indicate identical residues in the sequences.

the charybdotoxin-sensitive potassium channel were estimated from the inhibition of [^{125}I]charybdotoxin binding to rat brain crude synaptosomal (P_2) membrane vesicles. Briefly, [^{125}I]charybdotoxin (0.2 nM) was incubated with P_2 membranes (0.8 mg/ml) in the presence or absence of various concentrations of sapecin and sapecin B for 10 min at 25°C. The P_2 membranes were trapped on GF/C glass fiber filters and the radioactivity retained was determined by gamma counting.

3. Results

3.1. Synthesis of sapecin and sapecin B

Linear precursors of sapecin and sapecin B were synthesized by solid-phase methodology of Fmoc-chemistry. After TFA cleavage, diluted solutions of crude linear peptides were stirred slowly while exposed to air in the various conditions to form disulfide bonds. No remarkable change was observed by changing the pH of the solution, reaction temperature, and by addition of redox reagents. Only the peptide concentration strongly affected the yields of the products, since large amounts of precipitates formed at higher concentrations, suggesting the formation of polymeric products. The best results were obtained by the oxidation in 0.1 M ammonium acetate (pH 7.8) at a peptide concentration of 0.02 mM at room temperature. As shown in Fig. 2, the cyclization reaction of both sapecin and sapecin B afforded major products in this condition. These major products were purified until they showed as a single peak in HPLC analysis. The overall yields from starting resins were 5% for sapesin and 11% for sapecin B. Amino acid analysis of synthetic sapecin and sapecin B resulted in a good agreement with theoretical values (data not shown) and FAB-MS measurement gave $[M+H]^+$ ion peaks at 4,074.8 for sapecin and 3,998.0 for sapecin B in agreement with calculated values of 4,074.7 for sapecin and 3,997.8 for sapecin B. In addition, the disulfide pairings of synthetic sapecin B were confirmed by the method as described for native sapecin B [2].

3.2. Antibacterial activities of synthetic sapecin and sapecin B

Fig. 3 shows the inhibitory effects of sapecin and sapecin B on the growth of *Staphylococcus aureus* IFO 12732 used as an indicator bacterium. Synthetic sapecin was as potent as native sapecin. The activity of synthetic sapecin B was about 20-times less potent than sapecin, which agreed well with the results reported previously [2].

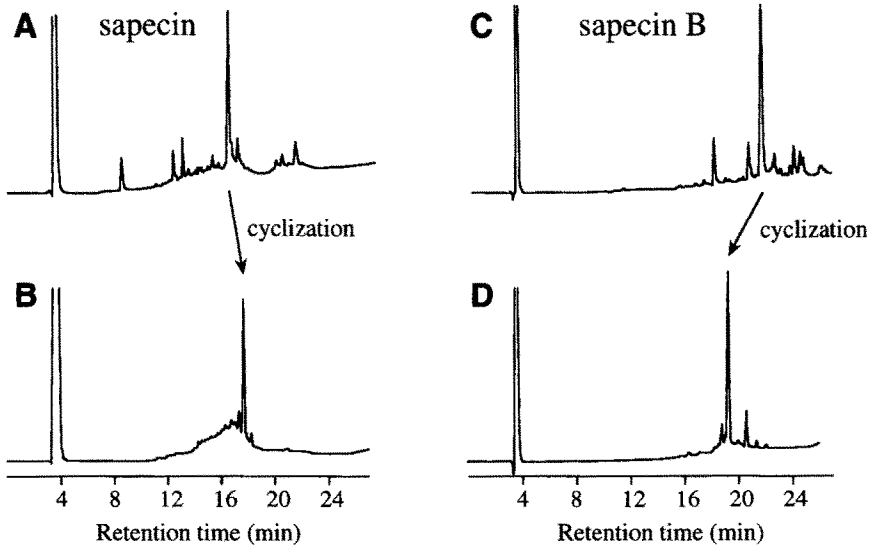

Fig. 2. HPLC profiles of the cyclization reaction of sapecin and sapecin B. (A) Crude linear sapecin, (B) crude cyclized sapecin, (C) crude linear sapecin B, (D) crude cyclized sapecin B. Column, Shim-pack CLC-ODS (4.6×250 mm; Shimadzu). Solvent system, linear gradient from 5% to 65% CH_3CN in 0.1% TFA for 30 min. Flow rate, 1 ml/min. Monitoring, absorbance at 230 nm (intensity is not to scale). Large peaks at around 4 min are due to the solvent used for the samples.

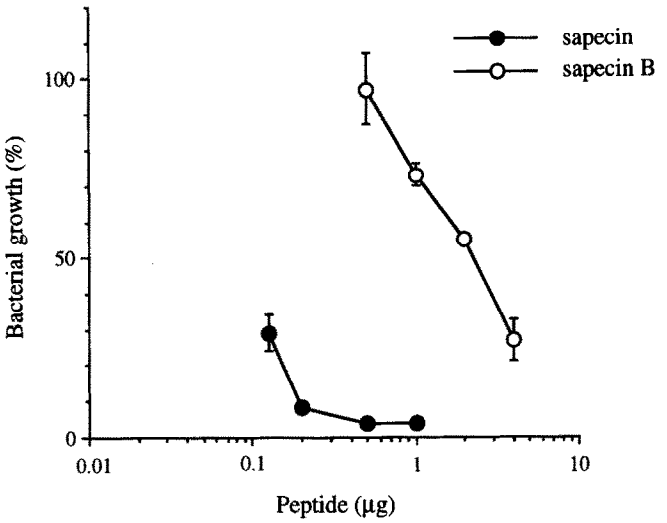

Fig. 3. Inhibitory effects of sapecin and sapecin B on the growth of *Staphylococcus aureus* IFO 12732.

Measurements of antibacterial activities of synthetic sapecin and sapecin B confirmed the structure and activity of sapecins.

3.3. *Effects of sapecins on charybdotoxin binding to the potassium channel*

Bontems et al. [6] pointed out that charybdotoxin, a potassium channel blocker isolated from a scorpion venom, shares striking structural similarities with some insect defencins, including sapecin. The distribution of cysteine residues and pairings of the three disulfide bonds in sapecins and charybdotoxin are almost identical (Fig. 1). In order to examine whether sapecins affect charybdotoxin binding to the potassium channel, we investigated the inhibitory effects of sapecin and sapecin B on the binding of ^{125}I-labeled charybdotoxin to rat brain synaptic membrane vesicles. As shown in Fig. 4, charybdotoxin completely suppressed [^{125}I]charybdotoxin binding to the basal level at 10^{-9} M concentration, while neither sapecin nor sapecin B showed inhibitory effects even at 10^{-6} M concentration. These results indicate that the high-affinity binding site for charybdotoxin on the potassium channel is not affected by sapecins.

4. Discussion

Synthesis of a cyclic peptide with multiple disulfide bonds is not as straightforward as that of a linear peptide. However, in many cases, controlled air oxidation of a linear precursor gives a single major compound with native-type disulfide pairings. Fortunately, cyclization reaction of sapecins afforded fully active peptides as major products in satisfactory yields, suggesting that these peptides took on a stable conformation.

The conformation of sapecin has been determined by ^{1}H NMR and dynamical simulated annealing calculations [7] and it was shown to be similar to that of charybdotoxin [6]. Although the conformation of sapecin B has not yet been determined, its overall structure is expected to be much more similar to that of charybdotoxin, because the numbers of the residues between the first and the second cysteine residues are 12 for sapecin, and 6 for sapecin B and 5 for charybdotoxin (Fig. 1). In addition, most of the basic residues in sapecin B are conserved in the sequence of charybdotoxin. Because basic residues in charybdotoxin were important for the interaction with the potassium channel [8–11], we suspected the possibility that sapecin B shared the same binding site as charybdotoxin. However, neither sapecin nor sapecin B bound the high-affinity binding site for charybdotoxin on the potassium channel. These results suggest that the nature and the local conformation of side chains are essential for the binding of charybdotoxin

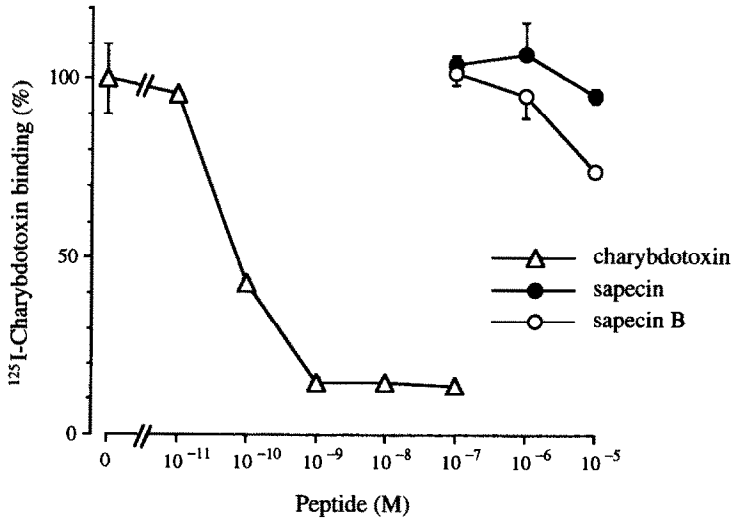

Fig. 4. Inhibitory effects of sapecin and sapecin B on [^{125}I]charybdotoxin binding to rat brain synaptic membranes.

rather than a globular conformation maintained by the conserved intramolecular disulfide framework.

Giangiacomo et al. [12] reported that the deletion of the N-terminal 6 amino acid residues in charybdotoxin significantly reduced the affinity. Therefore, it is likely that the N-terminal domain of charybdotoxin plays an important role in the binding to the potassium channel. Both sapecin and sapecin B have only 2 amino acid residues in this region (Fig. 1) and this may be one of the reasons why sapecins do not share the same binding site as charybdotoxin.

Recently, Shimoda et al. [13] reported that sapecin B inhibited part of the voltage pulse-induced potassium currents of rat cerebellar Purkinje cells. They suggested that this effect was due to inhibition of the calcium-activated potassium channel. In combination with the present result of the binding assay, sapecin B is expected to be a new probe for the discrimination of several binding sites on calcium-activated potassium channels. Large amounts of synthetic sapecin B will be useful for further study on the mechanism of action of this unique peptide.

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