Primary structure of a potassium channel toxin from the sea anemone *Actinia equina*

Sonomi Minagawa, Masami Ishida, Yuji Nagashima, Kazuo Shiomi*

Department of Food Science and Technology, Tokyo University of Fisheries, Konan-4, Minato-ku, Tokyo 108-8477, Japan

Abstract A potassium channel toxin (AeK) was isolated from the sea anemone *Actinia equina* by gel filtration on Sephadex G-50 and reverse-phase HPLC on TSKgel ODS-120T. AeK and α-dendrotoxin inhibited the binding of ^{125}I-α-dendrotoxin to rat synaptosomal membranes with IC_{50} of 22 and 0.34 nM, respectively, indicating that AeK is about sixty-five times less toxic than α-dendrotoxin. The complete amino acid sequence of AeK was elucidated; it is composed of 36 amino acid residues including six half-Cys residues. The determined sequence showed that AeK is analogous to the three potassium channel toxins from sea anemones (BgK from *Bunodosoma granulifera*, ShK from *Stichodactyla helianthus* and AsKS from *Anemonia sulcata*), with an especially high sequence homology (86%) with AsKS.

Key words: Amino acid sequence; Potassium channel toxin; Sea anemone; *Actinia equina*

1. Introduction

Sea anemones are well known to contain two classes of toxins (15–21 kDa hemolysins [1] and 3–5 kDa sodium channel toxins [1–4]) and about 6 kDa serine protease inhibitors mostly belonging to the Kunitz-type family [5–10]. Besides these biologically active substances, the following six potassium channel toxins were recently isolated from three species of sea anemones and characterized: BgK from *Bunodosoma granulifera* [11], ShK from *Stichodactyla helianthus* [12] and kaliseptine (AsKS) and three kalicludines (AsKC1–3) from *Anemonia sulcata* [13]. These toxins behave as blockers of voltage-sensitive potassium channels, similar to dendrotoxins from mamba snakes [14], mast cell degranulating peptide from the honeybee [15] and noxiustoxin from the Mexican scorpion [16]. Based on the determined amino acid sequences, they can be divided into two groups, group 1 toxins (BgK, ShK and AsKS) composed of 35–37 amino acid residues and group 2 toxins (AsKC1–3) of 58 or 59 residues. The group 1 toxins are a new family of potassium channel toxins in that their amino acid sequences are entirely different from those of the known voltage-sensitive potassium channel toxins from other sources, thereby being expected to be useful tools for studies on the structure and function of potassium channels. On the other hand, the group 2 toxins seem to be none other than Kunitz-type protease inhibitors. In fact, they have been shown to have high sequence homologies with the known Kunitz-type protease inhibitors and to exhibit antitryptic activity as well as potassium channel toxicity [13].

In our recent screening, the aqueous extract of *Actinia equina*, a relatively small species found in coastal waters of Japan, was established to display potassium channel toxicity [17]. None of the four Kunitz-type protease inhibitors so far discovered in *A. equina* were shown to be responsible for the potassium channel toxicity observed. Instead, a potassium channel toxin (named AeK) was isolated and demonstrated to belong to the group 1 family. The present paper deals with the isolation and primary structure of AeK from *A. equina*.

2. Materials and methods

2.1. Sea anemone

Specimens of *A. equina* were captured at Katsuura, Chiba Prefecture, in April 1997. They were transported alive in sea water to our laboratory and stored at −20°C until used.

2.2. Isolation procedure

Frozen samples (ten specimens, 33 g) were thawed and extracted with five volumes of distilled water. The extract was applied to a Sephadex G-50 column (2.5×95 cm; Pharmacia, Uppsala, Sweden), which was eluted with 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0). Fractions of 8 ml were collected and measured for absorbance at 280 nm and potassium channel toxicity. Toxin-containing fractions were combined and then subjected to reverse-phase HPLC on a TSKgel ODS-120T column (0.46×25 cm; Tosoh, Tokyo, Japan). Elution was achieved at a flow rate of 1 ml/min by a linear gradient of acetonitrile (0–17.5% in 60 min) in 0.1% trifluoroacetic acid (TFA). Peptides were monitored at 220 nm with a UV detector. The elute showing toxicity was manually collected and lyophilized. Thus, the dried material was used as the purified toxin (AeK) in subsequent experiments.

2.3. Assay for potassium channel toxicity

Potassium channel toxicity was indirectly assayed by competitive inhibition of the binding of ^{125}I-α-dendrotoxin, a potent potassium channel toxin from the green mamba *Dendroaspis angusticeps*, to rat synaptosomal membranes, essentially according to the method of Harvey et al. [18]. Rat brains were purchased from Funakoshi (Tokyo, Japan), α-dendrotoxin from Sigma (St. Louis, USA) and ^{125}I-α-dendrotoxin with a specific radioactivity of 2000 Ci/mmol from Amersham (Tokyo, Japan). Rat synaptosomal membranes were suspended in synaptosomal buffer (130 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 20 mM Tris-HCl, pH 7.4) at a concentration of 0.25 mg protein/ml. For competitive binding experiments, 0.2 ml of the synaptosomal membrane suspension was incubated with 0.04 ml of sample solution and 0.01 ml of ^{125}I-α-dendrotoxin (20 pM in the synaptosomal buffer) at room temperature for 30 min. Then, the membranes were collected by centrifugation and washed three times with 1 ml of the synaptosomal buffer containing bovine serum albumin (1 mg/ml). The radioactivity bound to the membranes was measured on a gamma counter (COBRA II; Packard, Meriden, USA). Non-specific binding (below 10%) was determined in the presence of 0.56 μM α-dendrotoxin, instead of sample, and subtracted from each datum.

2.4. Protein determination

Protein was determined by the method of Lowry et al. [19], using bovine serum albumin as a standard.

2.5. Molecular weight determination

The molecular weight of the isolated toxin was determined by matrix assisted laser desorption ionization/time of flight mass spectrometry (MALDI/TOFMS) with a Shimadzu/Kratos Kompact MALDI I instrument. Sinapinic acid was used as a matrix.

*Corresponding author. Fax: +81 (3) 5463 0669.
E-mail: shiomi@tokyo-u-fish.ac.jp

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2.6. Sequence analysis

Sequence analyses were performed by the Edman degradation method with an automatic gas-phase protein sequencer (LF-3400D TriCart with high sensitivity chemistry; Beckman, Fullerton, USA).

3. Results and discussion

A potassium channel toxin (AeK) inhibiting the binding of ^{125}I-α-dendrotoxin to rat synaptosomal membranes was isolated from *A. equina* by gel filtration on Sephadex G-50 and reverse-phase HPLC on TSKgel ODS-120T. In gel filtration, AeK was eluted between fractions 49 and 55 (Fig. 1A). Subsequent reverse-phase HPLC afforded two significant peaks at retention times of 49 min and 53 min, of which the latter peak contained AeK (Fig. 1B). Thus, 100 μg of AeK was obtained from 33 g of the starting material. In our binding experiments, AeK as well as α-dendrotoxin blocked the binding of ^{125}I-α-dendrotoxin to rat synaptosomal membranes in a dose-dependent manner (Fig. 2). The IC_{50} (50% inhibitory concentration) values were estimated to be 22 nM for AeK and 0.34 nM for α-dendrotoxin, implying that AeK is about sixty-five times less potent than α-dendrotoxin.

Analysis by a sequencer determined the complete amino acid sequence of AeK comprising 36 residues as shown in Fig. 3. No impurities were detected in each cycle of the sequencing, demonstrating the homogeneity of the isolated toxin. The accuracy of the determined sequence of AeK was supported by the following two criteria: (1) no increased amino acid residues appeared in and after the 37th cycle of the sequencing; and (2) the molecular weight (3954) of AeK calculated from the determined sequence was consistent with that (3954) estimated by MALDI/FOFMS.

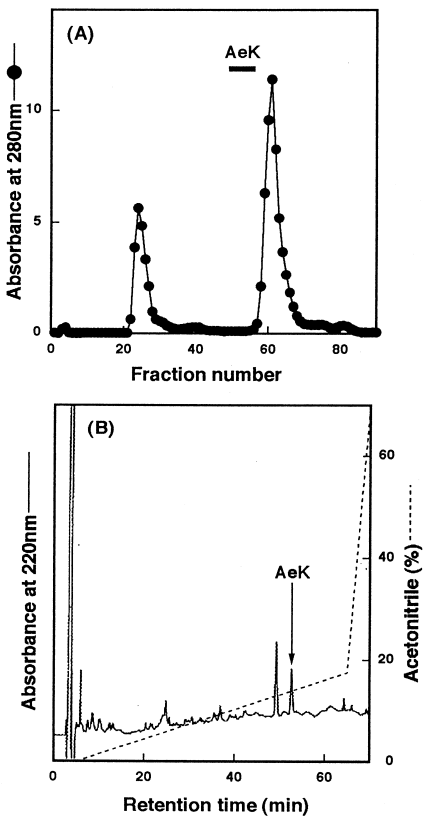

Fig. 1. Isolation of AeK. A: Gel filtration on Sephadex G-50. AeK-containing fractions are indicated by a bar. B: Reverse-phase HPLC on a TSKgel ODS-120T. AeK was eluted in the peak at a retention time of 53 min, as indicated by an arrow.

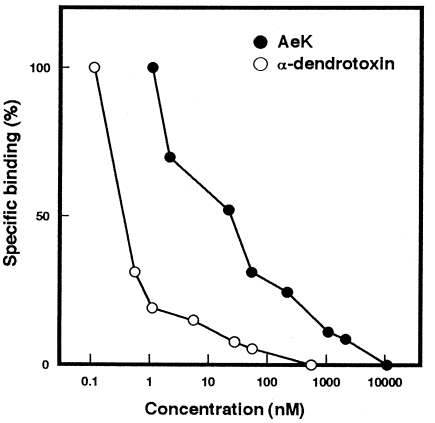

Fig. 2. Inhibition of the binding of ^{125}I-α-dendrotoxin to rat synaptosomal membranes by AeK and α-dendrotoxin. Each point represents the mean of two determinations.

As described in Section 1, the potassium channel toxins so far isolated from sea anemones are divided into two groups according to their chain length and sequence homology. Unequivocally, AeK is structurally analogous to BgK from *B. granulifera* [11], ShK from *S. helianthus* [12] and AsKS from *A. sulcata* [13], being a new member of the group 1 family (Fig. 3). The sequence homology of AeK with AsKS is as high as 86%, while those with BgK and ShK are moderate (53% with BgK and 36% with ShK). Importantly, AeK has six half-Cys residues at the same positions as the known group 1 toxins. This strongly suggests that the three disulfide bonds of AeK, though not assigned in this study, are located between Cys-2 and -36, Cys-11 and -29 and Cys-20 and -33, as previously reported for BgK [20] and ShK [21]. Furthermore, it is interesting to note that the two amino acid residues (Lys-22 and Tyr-23), which have been assumed to be the most essential residues for the binding of ShK with potassium channels [22], are conserved in AeK as well as BgK and AsKS.

Previous binding experiments, using both or at least one of ^{125}I-α-dendrotoxin and ^{125}I-dendrotoxin I as labeled toxins, confirmed that BgK [11,20] and ShK [12] are comparable in potassium channel toxicity to dendrotoxins, while the toxicity of AsKS is much less potent than dendrotoxins [13]. AeK, like AsKS, was shown to be remarkably less toxic than α-dendrotoxin in this study. This similarity in toxicity between AeK and AsKS coincides well with the fact that the sequence homology of AeK with AsKS is especially high compared to those with BgK and ShK. The low toxicity may be just what makes AeK as well as AsKS useful in studying the structure-activity relationship among the group 1 potassium channel toxins.

In relation to the sequence similarity between the group 2

Fig. 3. Alignment of the amino acid sequence of AeK with those of the known sea anemone potassium channel toxins. BgK from *Bunodosoma granulifera*; ShK from *Stichodactyla helianthus*; AsKS from *Anemonia sulcata*. The residues identical with AeK are boxed.

toxins (AsKC1–3 from *A. sulcata*) and Kunitz-type protease inhibitors, it should be pointed out that four Kunitz-type inhibitors have previously been isolated from *A. equina* and two of them completely sequenced [8]. None of the *A. equina* inhibitors, however, were obtained as potassium channel blockers in this study, suggesting that their toxicity, if any, is considerably weak as demonstrated for AsKC1–3 [13]. In order to discuss the biological function of Kunitz-type protease inhibitors, those from sea anemones including *A. equina* should be evaluated for potassium channel toxicity by using their pure preparation.

In conclusion, AeK isolated from *A. equina* is the fourth member of the group 1 potassium channel toxins from sea anemones. Our screening data [17] and those of Harvey et al. [23] suggest a wider distribution of potassium channel toxins in sea anemones. Further isolation and elucidation of sea anemone potassium channel toxins should be continued, since sea anemones may represent a new source of diverse potassium channel toxins.

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