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Functional expression and characterization of four novel neurotoxins from sea anemone *Anthopleura* sp.☆

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Abstract

The genes of four novel neurotoxins, named Hk2a, Hk7a, Hk8a, and Hk16a, were obtained from sea anemone *Anthopleura* sp. All four neurotoxins were composed of 47 amino acid residues and the variable residues among them were found in positions 14, 22, 25, and 37. To study their activities, the four toxins fused to the *Escherichia coli* thioredoxin were overexpressed by BL21 (DE3), cleaved off from the fusion partner, purified, and characterized with MALDI-TOF and CD assays. Contractile force studies of isolated SD atria indicated that rHk2a had the strongest and rHk7a the longest heart stimulation effect. Consequently, the Arg14, a highly conserved residue in various sea anemone neurotoxins, can be inferred to contribute to the duration but not the intensity of contraction-stimulating activity. Our work renders useful information to studies of sea anemone neurotoxins, especially to the clarification of the function of the disputative Arg14.

Keywords: Sea anemone; Neurotoxin; Fusion expression; Recombinant neurotoxin; Contractile force

Sea anemones are the most primitive ocean animals, which belong to phylum Cnidarians and class *Anthozoa*. The tentacles around the central mouth of sea anemone are important weapons to catch food and defend against their prey, and contain special stinging cells which can release a variety of biological active substances, including some potent toxins when stimulated by foreign objects physically and chemically [1,2]. Researchers have isolated two classes of toxins from different sea anemone species: neurotoxins and cytolysins. The neurotoxins are polypeptides consisting of 46–49 amino acid residues with three intra-molecular disulfide bridges [3] and having positive inotropic activity on cardiac muscle. The mechanism is to delay the inactivation and prolong the action potential of the fast sodium channel on the cardiac muscle cells by binding to specific receptor site 3 of the sodium channel [4,5]. Cytolysins are polypeptides whose molecular weights range from 15 to 20 kDa [6,7].

Up to now, many different neurotoxins have been isolated from different sea anemone species [8–10], such as Ap-A (Anthopleurin A), Ap-B, and Ap-C isolated by Norton and his co-workers [10–13]. Ap-B differs in 7 residues from Ap-A and has a heart stimulation effect about 12.5-fold stronger than that of Ap-A from *A. xanthogrammica* [11]. Functional studies of amino acid residues of sea anemone neurotoxins were carried out through chemical modification, comparison of amino acid sequence, site-directed mutagenesis, and the structural analysis. The results indicated that Arg-12 appeared to be involved in the ability to discriminate between neuronal and cardiac sodium channels [14] and Leu-18 is the most significant single contributor to the high affinity of Ap-B [15]. The analysis of three-dimensional structure of Ap-A and Ap-B revealed that Asp7

☆ *Abbreviations:* Ap-A, Anthopleurin A; Ap-B, Anthopleurin B; Ap-C, Anthopleurin C; TRX, thioredoxin; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; FPLC, fast performance liquid chromatography; MALDI-TOF-MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis. CD, circular dichroism.

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was close to the amino group of Lys37, together with the nearby residues Asp39 and His34, forming the pharmacophore of the neurotoxins [16,17].

The investigation of the three-dimensional structure of Ap-A and Ap-B indicated that they were constituted by four short strands of antiparallel β-sheet connected by three loops [16,17]. Structurally diversified neurotoxins isolated from several sea anemone species were powerful tools to study the structure and function of the voltage-gated sodium channels [4,18]. A diversified spectrum of toxins that could discriminate closely related receptor subtypes undoubtedly had a distinct evolutionary advantage [9]. On the other hand, the marked positive inotropic effect of sea anemone neurotoxin represented useful leads in the development of novel positive inotropic agents [10,19]. Thus, isolating different novel neurotoxins from different species of sea anemone is very useful to better understand their structure–function relationship of these molecules and to finally design therapeutic agents based on these molecules.

Anthopleura sp. is a common species in the South China Sea, and four genes encoding four novel neurotoxins were obtained from that species using molecular biology approach. These four neurotoxins fused with TRX were successfully expressed in *Escherichia coli* and the activities of these four neurotoxins on the isolated rat atria were studied. It was very interesting that the amino acid sequences of four novel neurotoxins differed in four places, which determined the different effects on heart stimulation.

Materials and methods

Bacterial strains and plasmids. Escherichia coli strain BL21 (DE3), (F^- *ompT* $hsdS_B$ (r_{B^-} m_{B^-}) *gal dcm* λ(*DE3*) (*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*)) and original plasmids pET22b were obtained from Novagen (Madison, WI, USA). *E. coli* strain DH5α, (*supE44* Δ*lacU169* (Φ*80 lacZ* Δ *M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA 1*) was supplied by Amersham Biosciences Corp., USA. Plasmid pTRX was reconstructed from pET22b (Peng, L., unpublished work; Fig. 1). Upstream primer 5′-AAACATATGATATGAGCGATAAAATTATTCACCTGAC-3′ and downstream primer 5′-AAAAAGCTTGGCCAGGTTAGCGTCGAG GA -3′ were used to amplify *TrxA* gene from the chromosome of *E. coli* strain K-12 via PCR. The PCR products were digested by *Nde*I/*Hin*dIII. The region coding for N-GlySerGlySerGly-C, N-6-his tag and multiple-cloning-site (MCS) was constructed by synthesis of two partially complementary sequences 5′-AAAAGCTTGGTTCTGGTTCTGGTCAT CATCATCATCATCATGGTACCGAATTCGGT-3′ and 5′-AAGCG GCCGCAAGCTTGGATCCAGAGATATCCTCGAGACCGAATT CGGTACCATGATG-3′ and elongation of them, then the products were digested by *Hin*dIII/*Not*I After double digestion of pET22b by *Nde*I/*Not*I, the linear plasmid was ligated with the two segments to obtain the final pTRX.

Chemical reagents, chromatography media, and columns. Tryptone and yeast extract were purchased from Oxoid England. Tris base, SDS, and IPTG were from Shanghai Sangon China. All other chemicals were of analytic grade from local manufacturers. One hundred millimolar IPTG stock solution was filter sterilized and 20% glucose (w/v) was autoclaved. Chelating Sepharose Fast Flow, Sephadex G-25 Fine and G-50 Fine were purchased from Amersham Pharmacia Biotech Ltd. Chromatography columns were products of a domestic manufacturer. FPLC was operated on Biologic LP from Bio-Rad Laboratories, USA.

Sample collection and preparation. Anthopleura sp. was collected from Zhanjiang, a coastal city by South China Sea. The detailed procedure about isolation of total RNA of tentacles, synthesis of tentacle first-stranded cDNA, PCR, and sequence of cDNA (3′,5′-RACE) was described previously [20].

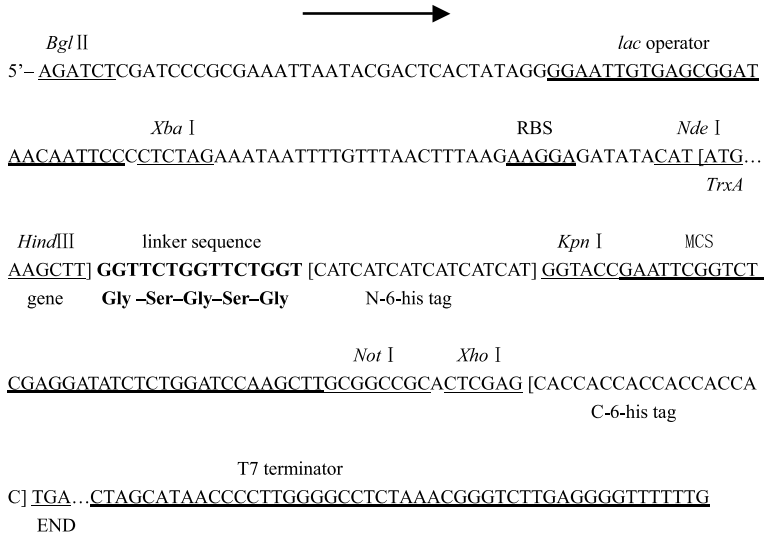

Fig. 1. pTRX (reconstructed from pET22b) cloning and expression region. RBS, ribosome binding site; MCS, multiple cloning site. The original plasmid pET22b was digested by *Nde*I and *Not*I and then ligated with *E. coli TrxA* gene and the linker sequence as described in 'Bacterial strains and plasmids.' In the amplified target genes by PCR, *Kpn*I restriction site followed by 3C protease cleavage site (5′-CTGGAAGTTCTGTTCCAG GGGCCC-3′) was introduced 5′ to the genes; while 3′ of them were extended with *Not*I restriction site. Subsequently the PCR products and pTRX were double digested by *Kpn*I and *Not*I mixed, and ligated. Routine screening and DNA sequencing techniques were carried out to obtain the properly cloned plasmids.

Construction of the fusion expression plasmid pTRX-Hk. The construction of fusion expression plasmid pTRX-Hk2a has been reported [20]. Other three fusion expression plasmids, pTRX-Hk7a, pTRX-Hk8a, and pTRX-Hk16a, were constructed according to the constructed procedure of pTRX-Hk2a. The 6× His tag was designed between thioredoxin and Hk gene fragment so as to make the fusion protein bond to the Ni^{2+} affinity chromatography column easy and to simplify the purification of fusion protein. Protease 3C cleavage site was designed between 6× His tag and Hk gene fragment for removing the fusion partner. The cleavage site of protease 3C was LEVLFQ ↓ GP. The recombinant sea anemone neurotoxin was obtained after cleavage by protease 3C takes two additional amino acid residues (G and P) in the N-terminus.

Expression and purification of four novel neurotoxins. Four recombinant sea anemone neurotoxins were over expressed in *E. coli* strain BL21 (DE3), which was often used as a host strain for the over expression of toxic proteins [21]. The fusion expression plasmid pTRX-Hk was transformed into BL21 (DE3). A single bacterial colony was used to inoculate 100 ml liquid LB medium supplemented with 100 μg/ml ampicillin. In order to reduce the basal expression level, 80% glucose was added to the liquid LB to a final concentration of 0.4%, and the LB medium was shaken at 37 °C about 15 h until the optical density at 600 nm (OD_{600}) approached to two. Three milliliters of the seed culture were added to 150 ml rich liquid LB medium (each liter containing 12 g tryptone, 6 g yeast extract, and 7 g NaCl). The culture was shake-incubated at 37 °C again for about 2.5 h until OD_{600} reached to 0.6, and then the cells were induced by addition of 100 mM IPTG to a final concentration of 0.1 mM, and at the same time, 20% glucose was added to a final concentration of 0.2% to supplement carbon source needed by the expression of the exogenous protein. After 10–11 h low temperature (21 °C) expression, cultures were harvested by centrifugation, 10 g of cell pellet were resuspended in 100 ml sonication buffer (50 mM Tris, 500 mM NaCl, and pH 7.0). The recombinant proteins pTRX-hk2a, pTRX-hk7a, pTRX-hk8a, and pTRX-hk16a were extracted by sonication. The cell debris was sedimented by centrifugation at 6000*g* for 40 min and the supernatant was used for purification.

The supernatant of the total cell extract was loaded onto Ni^{2+} chelating Sepharose fast flow column, which was charged with Ni^{2+} ions and pre-equilibrated by sonication buffer. The column was washed by the sonication buffer until the absorbent value at 280 nm remained constant. The flow-through fraction was pooled. The binding protein was eluted by 50 mM Tris buffer, pH 7.0, containing 500 mM NaCl and a series of imidazole concentrations increased step by step. Each fraction was collected and analyzed by tricine SDS–PAGE [22]. Fractions containing fusion proteins were applied to a Sephadex G-25 fine column and the buffer was changed to cleavage buffer, 50 mM Tris, 150 mM NaCl, and 1 mM EDTA, pH 8.0. DTT was added very slowly to the solution to a final concentration of 1 mM. The 3C protease produced by recombinant expression in our laboratory was added to the solution and cleavage reaction was carried out at 25 °C (water bathed) for 4 h. The cleaved protein was loaded on a Sephadex G50 Fine column (50 × 1000 mm) pre-equilibrated by 50 mM NH_4HCO_3. Each fraction was analyzed by tricine SDS–PAGE. Fractions containing the target proteins were pooled and lyophilized [CHRIST BETA 1-8K, Germany]. The lyophilized product was dissolved in ddH_2O and the concentration of the protein was determined using the method of Lowry [23], bovine serum albumin was used as the standard.

Determination of the molecular weights of four novel recombinant neurotoxins. The molecular weights of purified rHK2a, rHK7a, rHK8a, and rHK16a were determined by MALDI-TOF mass spectrometry (REFLEX III, Germany Bruker), sinapinic acid was used as a matrix, and the acceleration voltage was 25 kV.

Circular dichroism spectra of the recombinant neurotoxins. UV circular dichroism (CD) spectra were determined by J710 Spectropolarimeter (Japan) to investigate the folding of the recombinant neurotoxin. The CD-spectra of the recombinant neurotoxins were measured in the far-UV range (190–250 nm) at room temperature. Information on the toxins' secondary structure was deduced by the previously described method [24].

Effect of the recombinant neurotoxin on the isolated SD atria. The SD adult rats were killed by cervical dislocation. The atria was taken from thoracic cavity and mounted in 32 °C K–H buffer (NaCl 120 mM, KCl 4.8 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25.2 mM, $CaCl_2$ 2.5 mM, and Glucose 5.8) or low calcium K–H buffer which was the same as K–H buffer except for a 1/10 concentration of $CaCl_2$. The buffer was saturated continuously by mixture gas (95% CO_2, 5%O_2) to maintain constant pH. The isolated atria was tied to a LMB-2B isometric transducer and the rhythmic spontaneous beating was recovered. The contraction of the atria was recorded before and after applying the recombinant neurotoxins to the K–H buffer.

Results and discussion

Identification of four novel sea anemone neurotoxins

The preparation of total RNA from tentacles of *Anthopleura* sp. and the synthesis of first-strand cDNA have been described previously [20]. RT-PCR was performed using two primers A1 and A2, which were designed from the terminal amino acid sequence of Ap-B reported [20]. Four full-length cDNAs encoding four novel homologous neurotoxins, namely Hk2a, Hk7a, Hk8a, and Hk16a, respectively, were obtained and the sequences are shown in Fig. 2. The amino acid sequences of four new sea anemone neurotoxins were deduced by software DNATOOL5.1 as shown in Fig. 3. Each novel neurotoxin was composed of 47 amino acid residues. They had a high homology with Af I and Ap-C which also consisted of 47 amino acid residues [13,23].

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hk8   GGGGTCCCGTGCCTGTGCGACAGTGACGGTCCAAGCGTACGCGGCAATAC
hk16  GGGGTGCCGTGTTTGTGCGACAGTGACGGTCCAAGCGTGCGCGGCAATAC
hk7   GGGGTTCCATGTCTGTGTGACAGTGACGGTCCTAGCGTGCACGGCAATAC
hk2   GGAGTACCTTGCCTGTGCGACAGTGACGGCCCGAGCGTGAGAGGCAATAC

hk8   CTTATCAGGAACACTCTGGCTGTTCGGCTGCCCATCCGGTTGGCATAAC
hk16  CTTATCAGGGACACTCTGGCTGTTCGGCTGCCCATCCGGTTGGCATAAC
hk7   CTTGTCAGGGACAATCTGGTTGGCCGGCTGCCCATCCGGGTGGCATAAC
hk2   CTTGTCAGGGACACTCTGGCTGGCCGGCTGCCCATCCGGTTGGCATAAC

hk8    TGCAAGGCCCGTGGACCGACCATTGGCTGGTGCTGCAAGAAG
hk16   TGCAAGGCCCATGGACCGACCATTGGCTGGTGCTGCAAGAAA
hk7    TGCAAGGCCCATGGTCCGACCATTGGCTGGTGTTGCAAGAAA
hk2    TGCAAGGCCCATGGACCGACCATTGGCTGGTGCTGTAAGAAG
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Fig. 2. The sequence of full-length cDNA of four novel neurotoxins. The alphabets that are underlined indicate identity; the others indicate difference.

hk8 GVPCLCDSDGPSV*R*GNTLSGT*L*WL*F*GCPSGWHNCKA*R*GPTIGWCCKK

hk16 GVPCLCDSDGPSV*R*GNTLSGT*L*WL*F*GCPSGWHNCKA*H*GPTIGWCCKK

hk2 GVPCLCDSDGPSV*R*GNTLSGT*L*WL*A*GCPSGWHNCKA*H*GPTIGWCCKK

hk7 GVPCLCDSDGPSV*H*GNTLSGT*I*WL*A*GCPSGWHNCKA*H*GPTIGWCCKK

Fig. 3. The deduced amino acid sequences of four novel neurotoxins from the cDNA sequences. The italic alphabets indicate the mutation positions.

The homology of four novel sea anemone neurotoxins with other six neurotoxins is shown in Table 1. The amino acid sequence differences among Hk2a, Hk7a, Hk8a, and Hk16a were in positions 14, 22, 25, and 37. Most of the differences between four novel neurotoxins and known neurotoxins (ApA, ApB, ApC, Af I, As II, and As V) were also in these four positions. Arg in position 14 of Hk2a, Hk8a, and Hk16a was changed to His in Hk7a and Leu in position 22 of Hk2a, Hk8a, and Hk16a was changed to Ile in Hk7a. Ala in position 25 of Hk2a and Hk7a was changed to Phe in Hk8a and Hk16a and Arg in position 37 of Hk8a was changed to His in the other three neurotoxins.

In the same sea anemone species *Anthopleura* sp., four different genes encoding four similar neurotoxins were identified. Other sea anemone species such as *Anthopleura elegantissima* [8] and *Anthopleura anthogrammica* [9] also contain different but highly homologous neurotoxins within the same species, suggesting that in the course of long evolution, different but high homologous neurotoxins were produced by the sea anemone to perform different biological functions when capturing pray or defending against the predator. This might explain that homologous neurotoxins Ap-A and Ap-B were isolated from the same species *Anthopleura Xanthogrammic*a but the functions on the heart stimulation were different.

On the other hand, four genes encoding four novel neurotoxins acquired from sea anemone *Anthopleura* sp. added four new members to the sea anemone neurotoxin family, which could be useful for obtaining more information about the structure–function relationship of the neurotoxin and providing more leads for design of heart stimulant drugs. At the same time, more sea anemone neurotoxins found could provide even stronger tools to study the conformation of the proteins for their high specific affinity to sodium channel [5,18,25].

Table 1
The homology of four novel neurotoxins with six known neurotoxins

Homology (%)	Ap-A	Ap-B	Ap-C	Af I	As II	As V
Hk2a	89	76	95	95	91	89
Hk7a	85	72	91	95	91	85
Hk8a	87	74	91	91	87	85
Hk16a	89	76	93	93	89	87

Fusion expression in E. coli and purification of the recombinant neurotoxins

Four novel neurotoxins' sequences were cloned into the fusion expression vector pTRX along with the fusion partner thioredoxin. A region encoding the protease 3C recognition sequence was designed in the upstream of the neurotoxin for cleavage TRX and the 6× His tag before 3C sequence was added for convenience of purification. The vector pTRX-Hk was transformed into *E. coli* strain BL21(DE3) and the recombinant protein pTRX-Hk was over expressed in BL21(DE3). The total proteins and the supernatant of centrifugation post-sonication were analyzed by SDS–PAGE (15%), as shown in Fig. 4. The total proteins reflected the overexpression level of the recombinant protein in host strain and the supernatant was the soluble fraction of the total proteins. As indicated in Fig. 4, the target protein was highly expressed in BL21 with a good solubility.

The fusion protein was purified by the Ni^{2+} chelating Sepharose fast flow. With 50 mM Tris buffer which contains 500 mM NaCl and 300 mM imidazole, the fusion protein was eluted from the column, and other binding proteins were eluted at 100 mM imidazole concentration. The buffer of the fusion neurotoxin was changed to the cleavage buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, and pH 8.0) in G25 Sephadex column. DTT was added to a final concentration of 1 mM and the fusion protein was cleaved by incubation with protease 3C for 4 h at 25 °C. Pure recombinant neurotoxin was separated from the TRX fusion partner, undigested fusion protein, and other proteins by Gel

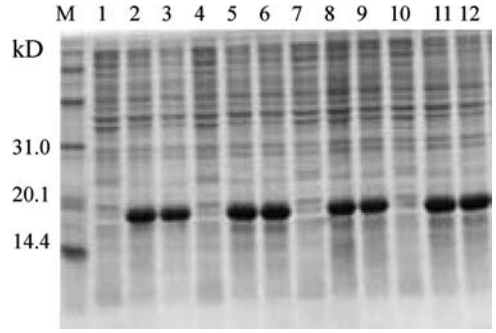

Fig. 4. SDS–PAGE analysis of four fusion protein (pTRX-Hk) expressed level in BL21(DE3). M, the standard protein marker; 1, total bacterial protein of BL21-pTRX-HK7a uninduced; 2, total bacterial protein of BL21-pTRX-HK7a induced by IPTG; 3, total soluble protein of BL21-pTRX-HK7a induced by IPTG; 4, total bacterial protein of BL21-pTRX-HK16a uninduced; 5, total bacterial protein of BL21-pTRX-HK16a induced by IPTG; 6, total soluble protein of BL21-pTRX-HK16a induced by IPTG; 7, total bacterial protein of BL21-pTRX-HK2a uninduced; 8, total bacterial protein of BL21-pTRX-HK2a induced by IPTG, 9, total soluble protein of BL21-pTRX-HK2a induced by IPTG; 10, total bacterial protein of BL21-pTRX-HK8a uninduced; 11, total bacterial protein of BL21-pTRX-HK8a induced by IPTG; 12, total soluble protein of BL21-pTRX-HK8a induced by IPTG.

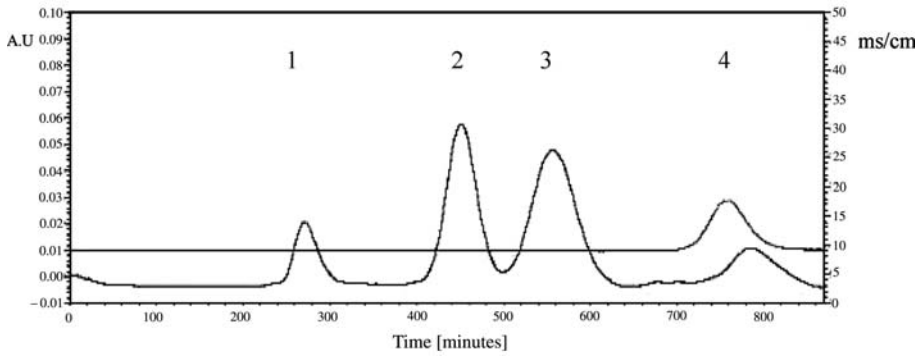

Fig. 5. Gel filtration chromatography of cleaved proteins on Sephadex G50 column of Hk8a. 1, the first fraction of G50: uncleaved fusion protein and anonymous proteins; 2, the second fraction of G50: TRX; 3, the third fraction of G50: purified recombinant neurotoxin hk8a; 4, the fourth fraction of G50 (DTT and the cleavage buffer were contained in this fraction).

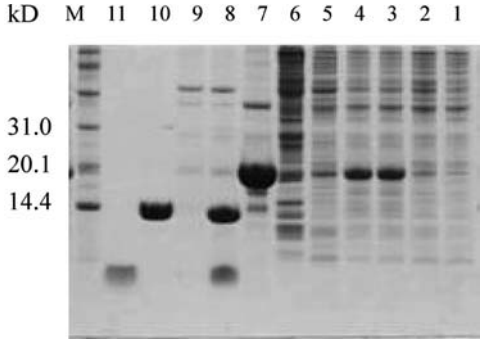

Fig. 6. Tricine SDS–PAGE analysis of expression and purity of Hk8a. 1, total bacterial protein of BL21-pTRX-Hk8a uninduced; 2, total bacterial protein of BL21-pTRX-Hk8a before induced by IPTG; 3, total bacterial protein of BL21-pTRX-Hk8a induced by IPTG; 4, total soluble protein of BL21-pTRX-Hk8a induced by IPTG; 5, the flow-through fractions of Ni^{2+} Chelating Sepharose chromatography; 6, washed protein from Ni^{2+} Chelating Sepharose chromatography by 100 mm imidazole; 7, purified fusion protein of TRX-Hk8a, eluted by 300 mm imidazole from Ni^{2+} Chelating Sepharose chromatography; 8, sample of the fusion protein TRX-Hk8a digested by Protease3C; 9, the first fraction of Sephadex G50 (uncleaved fusion proteins and anonymous protein); 10, the second fraction of Sephadex G50 (TRX); 11, the third fraction of Sephadex G50 (purified recombinant neurotoxin hk8a); M, the standard protein marker.

filtration Sephadex G-50 (50 mm × 1 m) (Fig. 5), and then lyophilized. Each fraction of the purification was analyzed by tricine SDS–PAGE (Fig. 6). Approximately 15 mg pure recombinant neurotoxin with biological activity could be obtained from 1 liter culture in our study. The yield of novel neurotoxins by this way was higher than that recombinant Ap-B obtained by Gene 9 fusion protein expression systems [26].

The molecular weight of the recombinant neurotoxin

The molecular weight of purified recombinant neurotoxin was measured by MALDI-TOF mass spectrometry. The measured molecular weights of purified recombinant neurotoxins Hk2a, Hk7a, Hk8a, and Hk16a were 5078.5, 4972.7, 4993.0, and 5010.6, respectively, which were similar to that (5KD) calculated from the amino acid sequence.

Circular dichroism

The refolding feature of the recombinant protein was obtained by the analysis of the protein far-UV CD

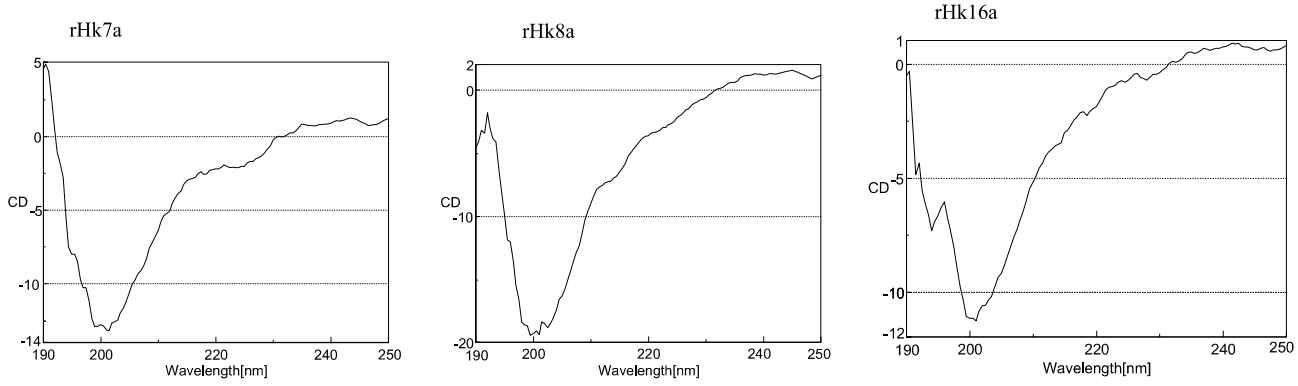

Fig. 7. Far-UV CD spectra of novel neurotoxins carried out at room temperature. rHk2a have been reported by Liu et al. [20]. The calculated secondary structures of rHk7a and rHk8a contain 57.7% β and 42.3 random; rHk16a contains 69.2 β and 30.8 random.

spectra. The result of CD and the relative secondary structure content of three recombinant neurotoxins are shown in Fig. 7. These data demonstrated that the secondary structures of all four novel neurotoxins were dominated by β-sheet with some random coil and no helix. These structures are identical with the basic sec-

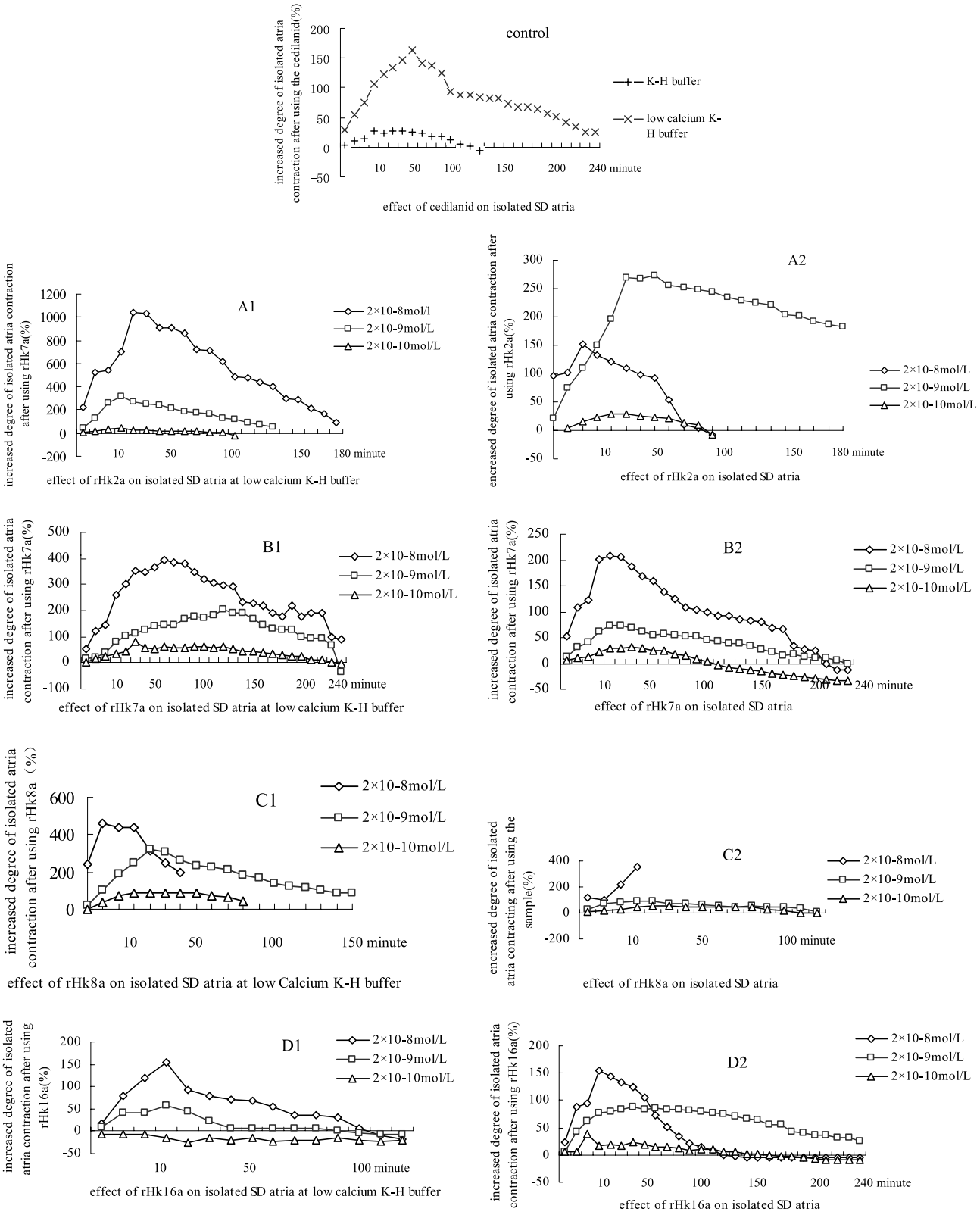

Fig. 8. The effect of the recombinant neurotoxins on the contractile force of isolated SD atria.

ondary structure of ApB, which predominantly consisted of the β structure [27]. Thus, we could conclude that the refolding of the recombinant neurotoxin was correct even with two additional residues.

Effect of the recombinant neurotoxin on isolated SD atria

The effects of recombinant neurotoxins on isolated SD atria were evaluated in the two models studying the contractile force. The first model was to estimate the increased contraction of the isolated spontaneous atria, which was mounted in K–H buffer. The second was to measure the increase of the heart failure atria in vitro. The heart failure model was obtained by exposing the isolated atria to low calcium K–H buffer, which could cause negative inotropism on the contraction of the atrium, as shown in Fig. 8. All the recombinant neurotoxins in our study could increase the contractile force of the isolated spontaneous SD atria and the heart failure atria in vitro.

As for the activity on the isolated heart failure atria, the neurotoxin rHk2a showed the strongest heart stimulation while rHk7a appeared to have the longest effect on the isolated heart failure SD atria. After applying the neurotoxin for 20 min, the increase of contractile force by rHk2a reached 1038% with 2×10^{-8} mol/L concentration and the heart stimulation was preserved for 180 min. rHk7a could maintain the cardiac status for 240 min at least with the same concentration. rHk16a displayed the least cardiac potency and rHk8a only preserved 40 min activity with 2×10^{-8} mol/L concentration. Compared with the effect on the isolated heart failure atria, the effect on the spontaneous isolated SD atria was relatively low. The contraction degree caused by the recombinant neurotoxin with 2×10^{-8} mol/L was higher than that caused by cedilanid at therapeutic concentration (2 μg/ml). From the concentration curve (2×10^{-8} mol/L, 2×10^{-9} mol/L, and 2×10^{-10} mol/L), it was concluded that four recombinant neurotoxins demonstrated the dose–response relationship.

Some key amino acid residues for the activity were confirmed by comparing the amino acid sequence with the activity of the neurotoxins. Four novel sea anemone neurotoxins (hk2a, hk7a, hk8a, and hk16a) found in our laboratory also included some key residues almost conserved in all neurotoxins: Asp7, Asp9, and Lys37 (Lys35 in four novel neurotoxins), which formed the pharmacophore. Leu18, the most significant single contributor to the high affinity of ApB, was also found in four novel neurotoxins. The variation of four novel neurotoxins with other known neurotoxins was in four positions: 14, 22, 25, and 37.

Arg14 is conserved in most known sea anemone toxins besides calitoxin, which lacks Arg14 instead of His14 [28]. The chemical modification results showed that Arg14 played an important role in the activity [1]. However, site-directed mutagenesis indicated that Arg14 was not required for the biological activity of ApB [19], suggesting that the function of Arg14 remains unclear. In our study, three of the four novel neurotoxins were found to have Arg in position 14 while Hk7a was replaced by His at the same position, and the activity of rHk7a on the contractile force of isolated SD atria is not the least potent while rHk7a could maintain the longest time to increase the heart stimulation. Thus, we concluded that Arg14 was not essential for the activity of sea anemone neurotoxins, but the duration of activity.

At the fourth changed position 37 (the equivalent position 39 in ApA and ApB), His residues and the replaced residue Arg are all cationic amino acids. Khera and Blumemthal [29] have concluded that neither of His39 nor His34 in ApB played a significant role in either folding or affinity for the channel, suggesting that the fourth changed position could not affect the activity of the novel neurotoxins. As for the function of amino acid residues in positions 22 and 25, further pharmacology and physiology studies were needed.

In summary, four cDNA clones encoding four homologous novel sea anemone neurotoxins were identified from Anthopleurin sp. These four genes were successfully expressed in *E. coli* by fusion expression with thioredoxin. All four recombinant neurotoxins could increase the contractile force of the isolated SD atria but the increase in degree was different among four neurotoxins. The analysis of the activity along with their amino acid sequences of four novel sea anemone neurotoxins indicated that Arg14 was not essential for the activity, but for the duration of activity.

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