Isolation and Amino Acid Sequences of Polypeptide Toxins in the Caribbean Sea Anemone *Condylactis passiflora*

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The aqueous extract of the Caribbean sea anemone *Condylactis passiflora* was potentially lethal to crabs and weakly hemolytic to animal erythrocytes but had no toxicity in mice. Three polypeptide toxins (named Cp I, II, and III) with lethal activity against crabs were isolated by ion-exchange chromatography on DEAE- and CM-cellulose, gel filtration on Sephadex G-50, and reverse-phase HPLC on Nucleosil 300-7C₁₈. The minimum lethal doses against crabs were estimated to be 7.3 $\mu\text{g/kg}$ (Cp I), 7.9 $\mu\text{g/kg}$ (Cp II), and 10 $\mu\text{g/kg}$ (Cp III). The amino acid compositions of the three toxins are closely related to each other; they are all rich in Asx, Ser, Gly, and half-Cys and are devoid of Met. In addition, it is noticeable that Cp I and II contain an unusual amino acid, hydroxyproline. The complete amino acid sequences of Cp I and II were determined. Both toxins have 47 amino acid residues, among which as many as 43 residues including all 6 half-Cys residues are homologous. Comparison with the sequences of the known sea anemone toxins reveals that Cp I and II are analogous to type 1 long neurotoxins but have some significant changes in their sequences.

Key words: sea anemone, *Condylactis passiflora*, polypeptide toxins, isolation, amino acid sequence

Sea anemones contain a variety of biologically active substances, typical of which are two classes of toxins, cytotoxins and sodium channel binding neurotoxins. Anemone cytotoxins are common basic proteins with molecular weights of 15000–21000.¹⁾ They possess potent hemolytic activity that is inhibited by sphingomyelin. On the other hand, sodium channel binding neurotoxins that prolong and inhibit the inactivation process of sodium currents during depolarization are polypeptides with molecular weights of 3000–5000.²⁾ They are further classified into three types, two (types 1 and 2) composed of long polypeptides containing 46–49 amino acid residues and one (type 3) of shorter polypeptides containing 27–31 residues. There is extensive sequence homology (>60%) either within the type 1 toxins, isolated from the genera *Anemonia*,^{3–5)} *Anthopleura*,^{6–8)} and *Bunodosoma*,⁹⁾ or within the type 2 toxins, isolated from the genera *Heteractis* (formerly *Radianthus*)^{10–15)} and *Stichodactyla* (formerly *Stoichactis*).¹⁶⁾ Although a little sequence homology (about 30%), such as the same locations of the 6 half-Cys residues, is seen between the type 1 and 2 toxins, both types of toxins are immunologically distinguished from each other. The type 3 neurotoxins, isolated from limited species of sea anemones,^{17–19)} have very little homology with the type 1 or 2 toxins.

Sea anemone toxins are relatively well characterized as described above. It is, however, possible that structurally and/or functionally interesting toxins differing from usual cytotoxins and neurotoxins will emerge in sea anemones, as in the cases of metridiolysin²⁰⁾ from *Metridium senile*, a cholesterol-inhibitable cytotoxin with a higher molecular weight, and calitoxin²¹⁾ from *Calliactis parasitica*, a poly-

peptide neurotoxin with less structural relationship to the type 1–3 toxins. In view of this possibility, we recently screened for toxins in several species of sea anemones. As a result, polypeptide toxins containing an unusual amino acid, hydroxyproline, were newly found in the Caribbean sea anemone *Condylactis passiflora*. Furthermore, the *C. passiflora* toxins were analogous to type 1 long toxins but had a number of alterations in the amino acid residues conserved for the known type 1 toxins. The present paper deals with the isolation and amino acid sequences of *C. passiflora* toxins.

It should be pointed out that the sea anemone used in this study was very similar to *Condylactis gigantea* but could not be identified. Anemone specimens from the Caribbean Sea, which are similar or identical to *C. gigantea*, are often identified in hobby literature as *C. passiflora*.²²⁾ For convenience, our sample is called *C. passiflora* throughout this paper, although the name of *C. passiflora* is not always recognized by taxonomists.

Materials and Methods

Sea Anemone

A live specimen (body weight about 60 g) of *C. passiflora*, which had been captured in the Caribbean Sea and imported to Japan, was purchased from Hikaru Aquarium (Tokyo), in October 1994. It was transported alive in sea water to our laboratory and stored at -20°C until use.

Assay of Toxicity

Specimens of the freshwater crab *Potamon dehaani* weighing about 5 g were purchased at the Tokyo Central

wholesale Market. Various dilutions of a given sample were intrahaemocoelically injected into groups of two crabs at the junction between the body and the leg. The injection volume was fixed at 10 μ l/g. Toxicity was judged to be positive when both crabs of each group were killed within 2 h after injection, and was expressed in terms of crab unit (CU), where 1 CU was defined as the minimum amount of toxin to kill a 5 g crab, or in terms of minimum lethal dose (μ g/kg).

Toxicity was also assayed using male mice (ddY strain) weighing about 20 g purchased from Sankyo Labo Service (Tokyo). Sample solutions were intravenously injected into mice at 10 μ l/g and the mice observed for 1 day.

Assay of Hemolytic Activity

Hemolytic activity against animal (cow, horse, sheep, and rabbit) erythrocytes was measured by the method of Shiomi *et al.*²³⁾ and expressed in hemolytic unit (HU), where 1 HU was defined as the amount of toxin causing 50% hemolysis.

Isolation Method

In each column chromatography described below, proteins were followed by absorbance at 280 nm and toxins by lethal activity to crabs.

The frozen sample of *C. passiflora* was thawed and macerated in a motor. Thirty grams of the macerated sample were extracted twice with 2 volumes of distilled water and the extract was dialyzed using a Spectra/Por 6 membrane (molecular weight cut off 1000; Spectrum Medical Industries, TX, USA) against 10 mM phosphate buffer (pH 7.0). The inner solution was applied to a DEAE-cellulose column (1.5 $\times$ 22 cm), which was eluted with 10 mM phosphate buffer (pH 7.0), followed by 1 M NaCl in the same buffer. Toxic fractions were combined and put onto a CM-cellulose column (1.5 $\times$ 22 cm), which was successively eluted with 10 mM phosphate buffer (pH 7.0) and 1 M NaCl in the buffer. Toxin-containing fractions were pooled and lyophilized. The dried material was dissolved in 5 ml of 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl and chromatographed on a Sephadex G-50 column (2.2 $\times$ 46 cm). Active fractions were combined and subjected to reverse-phase high performance liquid chromatography (HPLC) on a Nucleosil 300-7C₁₈ column (0.46 $\times$ 25 cm; Macherey-Nagel, Germany). Elution was achieved at a constant flow rate of 1 ml/min by a gradient (see Fig. 2 for detail) of acetonitrile in 0.1% trifluoroacetic acid (TFA). Thus, three toxins, named Cp I, II, and III, were isolated.

Protein Determination

Protein was determined by the method of Lowry *et al.*,²⁴⁾ using bovine serum albumin as a standard.

Amino Acid Analysis

Each isolated toxin (about 200 μ g) was hydrolyzed with 0.3 ml of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole in an evacuated tube at 115°C for 24 h. After hydrolysis, the solution was neutralized with an equal volume of 3.5 M NaOH and diluted with 1 ml of 0.2 M citrate buffer (pH 2.2). A 0.2 ml portion of the dilution was applied to an Atto MLC-703 amino acid analyzer.

S-Carboxamidomethylation of Cp II

Cp II (350 μ g) was dissolved in 1 ml of 0.5 M Tris-HCl buffer (pH 8.5) containing 6 M guanidine-HCl and 2.5 mM EDTA and reduced with 2 mg of dithiothreitol for 1 h at room temperature. Then, 20 mg of iodoacetamide was added and the reaction mixture was kept at room temperature for 1 h. The S-carboxamidomethylated (CAM) derivative produced was isolated by reverse-phase HPLC on a TSK ODS-80Ts column (0.46 $\times$ 15 cm; Tosoh, Tokyo), using a linear gradient of acetonitrile (0–70% in 60 min) in 0.1% TFA.

Enzymatic Degradation

Cp I and II (50 μ g each) were individually digested with 0.1 mU of asparaginylendopeptidase (EC 3.4.22.34; Takara Shuzo, Otsu) in 0.2 ml of 50 mM sodium acetate buffer (pH 5.0), containing 10 mM dithiothreitol and 1 mM EDTA, at 37°C for 24 h. CAM-Cp II (70 μ g) was digested with 2 μ g of trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (EC 3.4.21.4; Worthington Biochemical, NJ, USA) in 0.3 ml of 50 mM ammonium bicarbonate at 37°C for 22 h. The peptide fragments generated by each enzymatic digestion were separated by reverse-phase HPLC on the above TSK ODS-80Ts column using a gradient (see Figs. 3–5 for detail) of acetonitrile in 0.1% TFA.

Sequence Analysis

Sequence analysis was performed on an automatic gas-phase protein sequencer (LF-3400D TriCart with high sensitivity chemistry; Beckman, CA, USA). Cysteine was detected as PTH-dehydroalanine-dithiothreitol in the samples without S-carboxamidomethylation. Prior to analysis, the native sample (2 nmol) of Cp I or II was reduced in 10% 2-mercaptoethanol at 70°C for 60 min. Peptide samples (0.3–1 nmol) isolated from each enzymatic digest were directly applied to the sequencer.

Results

Toxicity

The crude extract of *C. passiflora* was potentially lethal to crabs (3840 CU/ml) but its intravenous injection into mice caused neither signs of illness nor death. The crabs challenged with lethal doses exhibited tonic paralysis in legs, followed by occasional convulsion, and died within 2 h. Due to tonic paralysis in legs, the crabs that were placed on their back became unable to return to their normal position at least within 30 min. In order to save time, therefore, toxicity was often checked by observing the crabs that were placed on their back 30 min after injection. In addition to the lethality to crabs, hemolytic activity was also displayed by the crude extract. However, since the estimated hemolytic activity ranging from 64 HU/ml (against rabbit erythrocytes) to 370 HU/ml (against cow erythrocytes) was rather weak probably due to a small amount of cytotoxins, isolation was directed only to the toxins having lethal activity against crabs.

Isolation of Toxins

The *C. passiflora* toxins were retained neither by DEAE-cellulose nor by CM-cellulose (data not shown). By pass-

ing through both DEAE- and CM-cellulose columns, about 80% of proteins contained in the crude extract were removed. When the active fractions obtained by ion-exchange chromatography were subjected to gel filtration on Sephadex G-50, three peaks with absorbance at 280 nm appeared; crab toxicity was detected only in the second peak (Fig. 1). Isolation of three toxins (Cp I, II, and III) was finally achieved by reverse-phase HPLC (Fig. 2). As summarized in Table 1, 30 g of the starting material afforded 2.1 mg of Cp I, 2.9 mg of Cp II, and 0.64 mg of Cp III, with a total recovery in toxicity of 25%. The minimum lethal doses against crabs were estimated to be 7.3 $\mu\text{g/kg}$ for Cp I, 7.9 $\mu\text{g/kg}$ for Cp II, and 10 $\mu\text{g/kg}$ for Cp III.

Fig. 1. Gel filtration of toxins.

Toxic fractions obtained by ion-exchange chromatography of the crude extract were applied to a Sephadex G-50 column (2.2 $\times$ 46 cm), which was eluted with 0.15 M NaCl in 10 mM phosphate buffer (pH 7.0). Fractions of 4 ml were collected.

Fig. 2. Separation of toxins by reverse-phase HPLC.

Toxic fractions obtained by gel filtration (see Fig. 1) were subjected to a Nucleosil 300-7C₁₈ column (0.46 $\times$ 25 cm). After washing with 0.1% TFA, the column was eluted with a gradient (represented by the dotted line) of acetonitrile in 0.1% TFA. The flow rate was maintained at 1 ml/min.

As shown in Table 2, the amino acid compositions of Cp I, II, and III were closely similar to one another. All toxins were rich in Asx, Ser, Gly, and half-Cys and lacked Met. However, there were some obvious differences among the three toxins. Cp I did not contain Tyr, while the other two toxins were devoid of Phe, instead of Tyr. Moreover, both Cp I and II contained an unusual amino acid, hydroxyproline, which was distinguishable from Cp III.

Amino Acid Sequences of Cp I and II

When the reduced sample of Cp I was analyzed by a sequencer, 37 amino acid residues from the N-terminus were identified. In order to determine the rest of the sequence, Cp I was digested with asparaginylendopeptidase. Two peptide fragments (I-N1 and I-N2) were isolated from the digest by reverse-phase HPLC (Fig. 3) and sequenced. In the case of Cp II, 28 N-terminal amino acid residues were identified by sequence analysis of the reduced sample. After digestion of the toxin with asparaginylendopeptidase, 2 peptides (II-N1 and II-N2) were isolated by reverse-phase

Table 1. Summary of toxin isolation

Isolation step	Protein (mg)	Total toxicity (CU)	Recovery in toxicity (%)	Minimum lethal dose ($\mu\text{g/kg}$)
Crude extract	387	5760×10^2	100	134
DEAE-cellulose	106	4860	84	44
CM-cellulose	73	4220	73	35
Sephadex G-50	21	3080	53	14
Nucleosil 300-7C ₁₈				
Cp I	2.1	572	10	7.3
Cp II	2.9	730	13	7.9
Cp III	0.64	128	2	10

Isolation of toxins was performed using 30 g of sample.

Table 2. Amino acid compositions of Cp I, II, and III

Amino acid	No. of residue* ¹		
	Cp I	Cp II	Cp III
Asx	4.9 (5)	6.2 (6)	6.0
Hyp* ²	1.0 (1)	0.9 (1)	0.0
Thr	3.0 (3)	2.1 (2)	2.0
Ser	4.7 (5)	4.8 (5)	4.8
Glx	3.3 (3)	3.5 (3)	3.4
Pro	1.1 (1)	1.0 (1)	2.3
Gly	6.6 (6)	6.9 (6)	6.9
Ala	1.1 (1)	2.1 (2)	2.1
Val	4.4 (4)	3.8 (4)	3.9
Cys (half)	5.0 (6)	4.9 (6)	4.3
Met	0.0 (0)	0.0 (0)	0.0
Ile	1.0 (1)	1.0 (1)	1.1
Leu	1.2 (1)	1.1 (1)	1.2
Tyr	0.1 (0)	1.9 (2)	2.1
Phe	1.0 (1)	0.0 (0)	0.0
His	3.5 (4)	1.7 (2)	1.7
Trp	2.2 (2)	2.1 (2)	1.9
Lys	1.9 (2)	2.0 (2)	2.0
Arg	1.0 (1)	0.9 (1)	1.1

*¹ Calculated on the basis that total residues are 47 for all toxins. The numbers in parentheses for Cp I and II are from the sequence analysis.

*² Hyp represents hydroxyproline.

Fig. 3. Reverse-phase HPLC of the peptides produced by digestion of Cp I with asparaginylendopeptidase.

Column, TSK ODS-80Ts (0.46 × 15 cm); elution, gradient (represented by dotted line) of acetonitrile in 0.1% TFA; flow rate, 1 ml/min; detection, absorbance at 220 nm.

Fig. 5. Reverse-phase HPLC of the peptides produced by digestion of Cp II with trypsin.

HPLC conditions as in Fig. 3.

Fig. 4. Reverse-phase HPLC of the peptides produced by digestion of Cp II with asparaginylendopeptidase.

HPLC conditions as in Fig. 3.

HPLC (Fig. 4) and subjected to sequencing. Furthermore, 1 peptide fragment (II-T1) was isolated from the tryptic

digest of CAM-Cp II by reverse-phase HPLC (Fig. 5) and also sequenced. Figure 6 shows the complete amino acid sequences of Cp I and II, together with the sequence data obtained for the native toxins and the enzymatic fragments. There was extensive high sequence homology (91%) between the two toxins, both of which were composed of 47 amino acid residues including 6 half-Cys residues. Both toxins had identical 35 amino acid residues from the N-terminus and identical locations of the 6 half-Cys residues, with only four changes at positions 36 (Thr/Asp), 38 (His/Tyr), 41 (Phe/Ala), and 42 (His/Tyr). In this study, the locations of the 3 disulfide bonds were not examined.

Discussion

The sea anemone *C. passiflora* was newly found to contain polypeptide toxins, together with a small amount of cytolytins. Three toxins were effectively isolated by a combination of ion-exchange chromatography, gel filtration, and reverse-phase HPLC. Although another minor toxin was suggested to exist by the elution profile in reverse-phase HPLC in which a small peak was observed between Cp I and II (Fig. 2), its purification was not carried out in this study. The toxins were all assumed to be very stable during the isolation procedure. Thus, the estimated mini-

Fig. 6. Amino acid sequences of Cp I and II.

The sequences of the N-terminal portion (native), asparaginylendopeptidase peptides (I-N1, I-N2, II-N1, and II-N2) and tryptic peptide (II-T1) were determined. Broken lines indicate the portions not analyzed. Hyp at position 3 in both toxins represents hydroxyproline.

Fig. 7. Amino acid sequences of type I long toxins from sea anemones.

The upper two lines show the sequences of toxins (Cp I and Cp II) from *Condylactis passiflora* under study. Hyp at position 3 in both toxins represents hydroxyproline. Other sequence data of toxins were modified from Norton²⁾ and Malpezzi.⁹⁾ The residues which are boxed and those which are shaded were previously reported to be identical and conservatively substituted, respectively, by Norton.²⁾ AFT I and AFT II are obtained from *Anthopleura fuscoviridis*; ATX I, ATX II, and ATX V from *Anemonia sulcata*; AP-A and AP-B from *Anthopleura xanthogrammica*; AP-C from *Anthopleura elegantissima*; Bc III from *Bunodosoma caissarum*.

lum lethal doses against crabs of the isolated toxins (7.3–10 µg/kg) are comparable to the values reported for other sea anemone neurotoxins.²⁾ In contrast, the toxins are much less toxic or substantially non-toxic to mice, since intravenous injection of the crude extract exhibits no toxicity in mice. This high crustacean but low mammalian toxicity has also been recognized for some sea anemone neurotoxins such as ATX I of *Anemonia sulcata* and Sh I of *Stichodactyla helianthus*.²⁾

The amino acid compositions of *C. passiflora* toxins are similar to those of the four toxins isolated from another *Condylactis* species (*C. aurantiaca*).²⁵⁾ However, our toxins are devoid of Met as in the case of almost all neurotoxins from sea anemones, while those of *C. aurantiaca* are not. With respect to the amino acid composition of *C. passiflora* toxins, it is especially worth mentioning that Cp I and II contain an unusual amino acid, hydroxyproline. Although this amino acid is abundant in collagens and also is known to be a constituent of conotoxins,²⁶⁾ polypeptide neurotoxins isolated from the cone shell *Conus geographus*, it is not contained in the sea anemone polypeptide toxins known to date. In this connection, it is in-

teresting to note that our preliminary experiments also suggest the occurrence of hydroxyproline in a polypeptide toxin from another sea anemone *Heteractis (Radianthus) crispus* (unpublished data). Hydroxyproline may be more widely distributed in sea anemone toxins than expected.

Our determinations of the amino acid sequences for Cp I and II are reliable, even without confirmation of the C-terminal sequence by any specific method such as the carboxypeptidase method. It is obvious that the number of each amino acid obtained from sequence analysis agrees well with that estimated from amino acid analysis, only with a slight difference for Gly and half-Cys in both toxins (Table 2). Furthermore, the I-N1 and II-N1 fragments produced by treatment with asparaginylendopeptidase, having Glu as the C-terminal residue, are easily assignable to be the C-terminal peptides of Cp I and II, respectively, since the enzyme specifically cleaves the peptide bond at the carboxyl site of Asn. In the case of Cp I, the I-N2 fragment accounts for the gap between the sequence of 37 residues from the N-terminus and that of I-N1 (C-terminal peptide), although it is produced as a result of incomplete digestion, that is, the peptide bond between Asn-39 and

Ile-40 is not cleaved. On the other hand, in the case of Cp II, the two enzymatic fragments, II-N2 and II-T1, apparently make amends for the portion (between Ser-29 and Asn-39) which has remained unknown after analyses of the native sample and the fragment II-N1.

It is obvious that the *C. passiflora* toxins have high sequence homology with the type 1 long toxins isolated from other species of sea anemones. For comparison, together with the sequences of Cp I and II, those of the following type 1 toxins determined to date are shown in Fig. 7: AFT I⁶ and AFT II⁶ isolated from *Anthopleura fuscoviridis*, ATX I,³ ATX II⁴, and ATX V⁵ from *Anemonia sulcata*, AP-A⁷ and AP-B⁸ from *Anthopleura xanthogrammica*, AP-C² from *Anthopleura elegantissima* and Bc III⁹ from *Bunodosoma caissarum*. Since the locations of the 6 half-Cys residues are invariable in all toxins, it is relevant to consider that the 3 disulfide bonds involved in both Cp I and II are conservatively located between Cys-4 and -44, Cys-6 and -34, and Cys-27 and -45, as reported for AFT I and II⁶ and ATX I.³ Previously, Norton² compared 8 kinds of type 1 toxins (AFT I, AFT II, ATX I, ATX II, ATX V, AP-A, AP-B, and AP-C) as to their amino acid sequences and pointed out that 27 amino acid residues are homologous and 3 residues are conservatively substituted throughout all toxins. For Bc III, only two alternations are found in the homologous amino acid residues, Leu-5 being replaced by Arg and Ser-19 by Thr. On the other hand, both Cp I and II have a number of changes in either homologous or conservatively substituted amino acid residues, viz. Leu-5 to Arg, Arg-14 to His, Gly-26 to Ser, Pro-28 to Ala, Asn-33 to Lys, Ile-41 to Phe (Cp I) or Ala (Cp II), Gly-42 to His (Cp I) or Tyr (Cp II), and Trp/Tyr-43 to Glu. Thus, the homology of Cp I or II with other type 1 toxins is rather low (47–60%), compared to the statement of Norton² that there is more than 60% homology within type 1 toxins. Of the observed changes, the replacement of Arg-14, the only residue being clearly functional,² by His is of particular importance.

In conclusion, the *C. passiflora* toxins are structurally unique compared with the known type 1 neurotoxins; they contain an unusual amino acid, hydroxyproline, and have many changes, including the significant replacement of Arg-14 by His, in their sequences. Though not confirmed, it is very likely that the *C. passiflora* toxins act essentially in the same manner as the known type 1 toxins. Valuable information concerning the structure-function relationship of type 1 toxins will be provided by future study with the *C. passiflora* toxins.

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