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Amino acid sequence of a cytotoxin-like basic protein with low cytotoxic activity from the venom of the Thailand cobra *Naja naja siamensis*

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A cytotoxin-like basic protein (CLBP) was isolated from the venom of the Thailand cobra *(Naja naja siamensis)*. The cytotoxicity of CLBP toward FL cells was one order of magnitude lower than those of cytotoxins. The amino acid sequence was determined by a combination of conventional methods. The total number of amino acid residues was 62, giving a molecular mass of 6977 Da. The sequence at residues 25–30 in the CLBP molecule was found to be significantly different from those of cytotoxins. This region might play an important role in the cytotoxic activity of cytotoxins.

Cytotoxin; Cardiotoxin; Cytotoxicity; Amino acid sequence; Cytotoxin-like basic protein; (FL cell, Cobra venom)

1. INTRODUCTION

Cobra venom cytotoxins (cardiotoxins) are highly basic polypeptides consisting of 60–61 amino acid residues, and exhibit cytotoxic activities against many kinds of cells such as Yoshida sarcoma cells and Fogh-Lund (FL) cells. Recently, a cardiotoxin-like basic polypeptide (CLBP) and a less cytotoxic basic polypeptide (LCBP) were isolated from the venoms of the Formosan cobra *(Naja naja atra)* [1] and the Indian cobra *(N. naja)* [2], respectively. These proteins had amino acid sequences similar to those of cytotoxins although their cytotoxic activities were much lower than those of cytotoxins. Here, we isolated a cytotoxin-like basic protein, designated CLBP, from the venom of the Thailand cobra *(N. naja siamensis)*, determined the amino acid sequence, and compared the sequence with those of cytotoxins.

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Abbreviations: HPLC, high-performance liquid chromatography; PTH-, phenylthiohydantoin derivative; TLC, thin-layer chromatography

2. MATERIALS AND METHODS

2.1. *Isolation of toxins from cobra venom*

The lyophilized venom of *N. naja siamensis* was obtained from Miami Serpentarium (USA). Toxins were separated by gel filtration on Sephadex G-75 followed by CM-cellulose column chromatography as described [3]. 1 9 venom was dissolved in 1% acetic acid and applied to a Sephadex G-75 column equilibrated with the same solvent. The fraction containing proteins of about 5–10 kDa was lyophilized and subjected to CM-cellulose column chromatography, the column having been equilibrated with 0.1 M sodium acetate (pH 6.0). Toxins were eluted with a linear gradient from 0.1 M sodium acetate (pH 6.0) to 0.5 M sodium acetate buffer (pH 6.5). Each fraction obtained was desalted on a Sephadex G-25 column and lyophilized. The homogeneity was confirmed by reversed-phase HPLC on a SynChropak RP-8 col-

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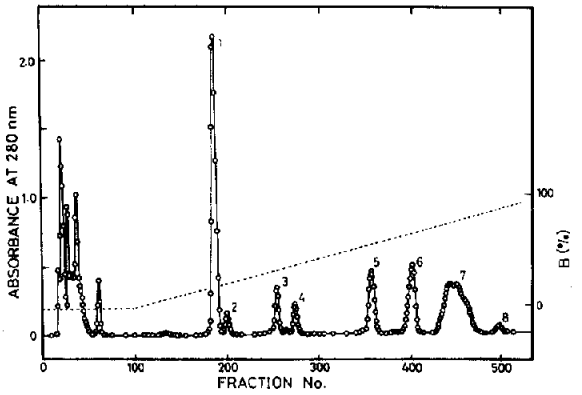

Fig.1. CM-cellulose column chromatography of the fraction obtained from Sephadex G-75 column chromatography. A CM-cellulose column (2.7×90 cm) was equilibrated with 0.1 M sodium acetate buffer (pH 6.0) and the toxins were eluted by a linear gradient from solution A, 0.1 M sodium acetate buffer (pH 6.0), to solution B, 0.5 M sodium acetate buffer (pH 6.5). Fractions of 13 ml each were collected at a flow rate of 62 ml/h.

umn (SynChrom, USA) with 0.1% trifluoroacetic acid containing a linear gradient from 0 to 45% acetonitrile. *S*-carboxymethylated (Cm) derivatives of cytotoxin and cytotoxin-like basic protein were prepared by the method of Crestfield et al. [4].

2.2. *Cytotoxicity toward FL cells*

FL cells were suspended in phosphate-buffered saline (PBS) at a concentration of 2.5×10^6 cells/ml. Various concentrations of each protein fraction were added separately to the cell suspensions and the mixtures were incubated at 37°C for 30 min. The cytotoxic activity was measured by the

Table 1

Amino acid compositions and cytotoxicities of the fractions obtained from the CM-cellulose chromatography shown in fig.1

Amino acid	P-1	P-2	P-3	P-4	P-5	P-6	P-7-1	P-7-2	P-7-3	P-8
Cys	n.d.(10)	n.d.	n.d.	n.d.	7.6	n.d.	8.2	8.4	8.5	7.8 (8)
Asp	9.0 (9)	7.9	8.1	8.0	7.8	5.2	7.4	7.9	6.0	7.1 (7)
Thr	8.6 (9)	10.0	6.1	7.4	2.9	2.8	2.3	2.9	3.0	4.0 (4)
Ser	2.9 (3)	2.9	3.6	3.5	2.8	1.8	1.2	1.8	1.7	2.0 (2)
Glu	1.1 (1)	8.1	6.3	7.1	0.1	5.7	0.1	0.0	0.8	2.2 (2)
Pro	6.4 (6)	2.7	3.1	2.1	4.1	3.8	5.3	4.3	4.8	4.8 (5)
Gly	4.1 (4)	7.4	5.1	6.9	2.1	4.0	2.2	2.1	2.2	2.3 (2)
Ala	3.2 (3)	0.4	0.7	0.1	2.0	1.1	4.9	2.8	1.8	3.0 (3)
Val	3.5 (4)	1.4	2.0	1.1	3.7	1.1	4.7	5.0	5.5	1.3 (1)
Met	0.0 (0)	0.0	0.1	0.0	2.6	2.0	2.0	2.0	1.7	0.0 (0)
Ile	4.6 (5)	0.5	1.9	2.0	3.8	3.9	1.9	1.9	1.3	2.8 (3)
Leu	1.0 (1)	2.1	2.2	1.1	6.0	4.3	6.3	6.2	5.7	6.8 (7)
Tyr	1.0 (1)	2.1	1.2	2.0	1.3	2.1	2.9	3.1	2.9	1.9 (2)
Phe	3.0 (3)	0.4	0.3	0.1	1.0	3.1	1.1	1.1	1.9	2.9 (3)
Lys	4.7 (5)	3.5	5.4	2.2	7.6	4.8	9.0	8.5	8.3	10.6(11)
His	0.9 (1)	2.0	1.9	1.9	0.2	1.0	0.2	0.1	0.3	1.1 (1)
Arg	4.8 (5)	6.3	4.2	6.9	2.0	6.5	2.2	2.0	2.5	1.2 (1)
Trp	n.d. (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d. (0)
Total	71	-	-	-	-	-	-	-	-	62
50% cytotoxicity (μg/ml)	>100	>100	>100	>100	5.4	>100	10.6	16.1	12.6	>100
Toxins	NT	NT	NT	NT	CT-I	NT	CT-II	CT-III	CT-IV	CLBP

The values in parentheses of fractions P-1 and P-8 were based on the amino acid sequence cited from [9] and that determined in this study, respectively. Cysteine was determined as carboxymethylcysteine. n.d., not determined; NT, neurotoxin; CT, cytotoxin; CLBP, cytotoxin-like basic protein

trypan-blue exclusion test as in [5]. Cytotoxicity was expressed as ED_{50}: the protein concentration required to cause lysis of 50% of the cells.

2.3. *Amino acid analysis*

The protein and peptide samples were hydrolyzed with 6 N HCl containing 0.2% phenol at 110°C for 24 h in evacuated sealed tubes. The amino acid compositions were determined with an amino acid analyzer (Hitachi model L-8500).

2.4. *Studies on the amino acid sequence of CLBP*

1 mg Cm-CLBP was digested in 0.1 M NH_4HCO_3 overnight at 40°C with 20 μg staphylococcal protease (Miles Scientific, USA) or TPCK-trypsin (Sigma, USA). The peptides thus obtained were separated by reversed-phase HPLC on a Cosmosil 5C18-300 column (Nakarai, Japan) with 0.1% trifluoroacetic acid containing a linear gradient from 0 to 70% acetonitrile. The manual Edman degradation method was used to determine the N-terminal sequences of Cm-CLBP and peptides. PTH-amino acids were generally identified by HPLC [6], and in some cases by TLC [7]. The C-terminal sequence of Cm-CLBP was determined by carboxypeptidase digestion [8]: the protein sample was digested by carboxypeptidase A (Sigma) at 40°C for 120 min in 0.1 M Tris-HCl buffer (pH 8.0) and the amino acids released were determined with the amino acid analyzer.

3. RESULTS

3.1. *Isolation of toxins from Thailand cobra venom*

Fig.1 shows the CM-cellulose column chromatographic profile of the protein fraction obtained from Sephadex G-75 column chromatography of the *N. naja siamensis* venom. As judged from the results of reversed-phase HPLC, fractions P1–P-6 and P-8 were homogeneous, but this was not the case for fraction P-7. The latter fraction was found to be composed of three subfractions (P-7-1, P-7-2, P-7-3) that had peaks at fraction nos 434, 445 and 465, respectively. Table 1 shows the amino acid compositions and the cytotoxicities toward FL cells of all these protein fractions. The amino acid composition of fraction P-1 agreed with that of the long neurotoxin already sequenced [9]. Fractions P-2, P-3, P-4 and P-6 were assigned to the short neurotoxin or its homologues, since their amino acid compositions were very similar to those of the short neurotoxins [10], whereas fractions P-5, P-7-1, P-7-2 and P-7-3 were assigned to the cytotoxins, since they showed high cytotoxic activities. These cytotoxins were designated as cytotoxin I (CT-I), II (CT-II), III (CT-III), and IV (CT-IV), respectively. Sequence studies on these cytotoxins are now in progress. Fraction P-8 showed low cytotoxic activity, though its amino acid composition was similar to those of the cytotoxins. Similar cytotoxin-like basic proteins (CLBP) had been already isolated from the venoms of the Formosan cobra *(N. naja atra)* [1] and the Indian cobra *(N. naja)* [2].

3.2. *Amino acid sequence of cytotoxin-like basic protein*

Fraction P-8 was *S*-carboxymethylated, and the N-terminal sequence of this Cm-CLBP was determined to be the following: H-Leu-Lys-Cmc-His-

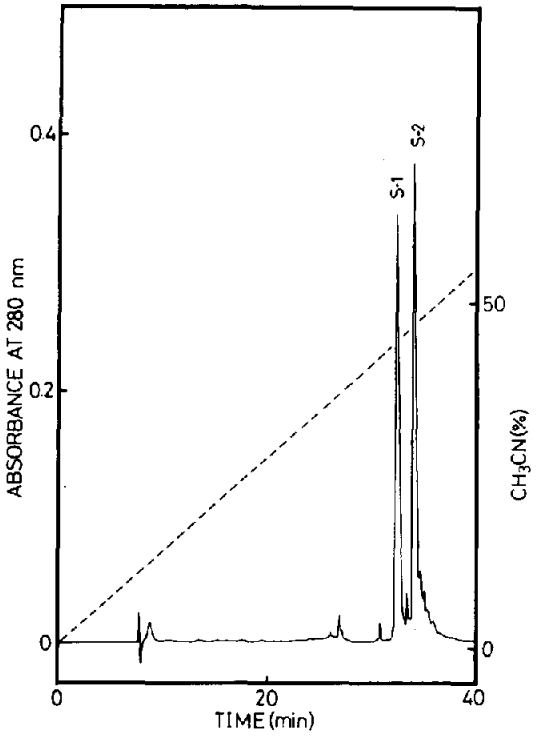

Fig.2. Separation of the staphylococcal protease digest of Cm-CLBP. The digest was fractionated by HPLC as described in section 2.

Table 2

Amino acid compositions of staphylococcal protease peptides (S-1, S-2) and tryptic peptides (T-1–T-9) of Cm-CLBP

Amino acid	S-1	S-2	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9
Cmc	2.18(2)	6.45(6)		0.75(1)	0.92(1)	1.06(1)			1.99(2)		2.81(3)
Asp	1.18(1)	5.91(6)		0.79(1)	0.85(1)	1.02(1)			1.93(2)	1.04(1)	1.78(2)
Thr	1.77(2)	2.16(2)		0.81(1)			0.93(1)	0.12			0.96(1)
Ser	0.15	1.77(2)						0.13		0.81(1)	0.81(1)
Glu	1.86(2)	0.30		0.75(1)	0.95(1)						
Pro	2.00(2)	3.34(3)		1.01(1)	0.87(1)			1.72(2)	0.85(1)		
Gly	0.12	1.92(2)			0.98(1)			0.14	0.99(1)		
Ala	0.12	2.88(3)					0.99(1)		0.96(1)	0.96(1)	
Val	0.19	1.15(1)									0.89(1)
Met											
Ile	0.91(1)	1.95(2)		0.83(1)				1.78(2)			
Leu	1.97(2)	5.00(5)	1.01(1)	1.35(1)		0.98(1)	0.97(1)	1.11(1)		1.96(2)	
Tyr	0.93(1)	1.05(1)		0.71(1)							0.95(1)
Phe	0.95(1)	2.01(2)		0.93(1)		0.99(1)		1.05(1)			
Lys	2.15(2)	9.14(9)	0.99(1)	0.73(1)	1.06(1)	1.01(1)	1.03(1)	2.83(3)	1.09(1)	1.02(1)	1.11(1)
His	0.88(1)	0.15		0.62(1)							
Arg		1.07(1)					0.10	0.89(1)			
Total	17	45	2	11	6	5	4	10	8	6	10
Yield (%)	25.5	19.8	35.0	9.7	34.8	41.6	26.1	25.5	13.5	45.2	19.9

Values in parentheses were taken from the sequence

Asn-Thr-Gln-Leu-Pro-Phe-Ile-Tyr-Lys-Thr-Cmc-Pro-Glu-Gly-Lys-Asn-Leu-Cmc-Phe-Lys-Ala-Thr-Leu-Lys-Lys-Phe-Pro-Leu-. Carboxypeptidase A released asparagine (0.7 mol/mol protein) and carboxymethylcysteine (0.16 mol/mol protein) after a 120 min incubation, suggesting the C-terminal residues of Cm-CLBP to be -Cmc-Asn-COOH. The staphylococcal protease digest of Cm-CLBP was subjected to HPLC (fig.2). The amino acid compositions of the two separated peptides, S-1 and S-2, are shown in table 2. The C-terminal fragment, S-2, was subjected to manual Edman degradation. The tryptic digest of Cm-CLBP was separated by HPLC into nine fragments, T-1–T-9, and their amino acid compositions were determined (table 2). The sequence studies on Cm-CLBP described above are summarized in fig.3. The total number of amino acid residues was 62, giving a molecular mass of 6977 Da.

When the amino acid sequence of CLBP was compared with those of cytotoxins, remarkable amino acid replacements were observed at residues 25–30, where hydrophobic amino acid residues in cytotoxins were replaced by hydrophilic ones in CLBP. This region might play an important role in the cytotoxic activity of cytotoxins.

The determined sequence of CLBP of *N. naja siamensis* differed from those of CLBP of *N. naja atra* [1] and LCBP of *N. naja* [2] in nine amino acid residues: the residues at 25, 26, 27, 28, 29, 30, 31, 34 and 36. The sequence Ala-Thr-Leu-Lys at positions 25–28 of the *N. naja siamensis* CLBP

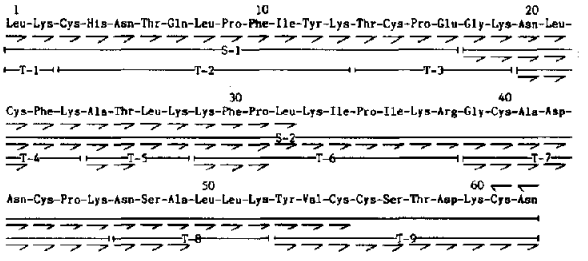

Fig.3. Summary of the sequence studies on CLBP. S- and T- refer to the staphylococcal protease peptides and tryptic peptides, respectively. The amino acid sequence was determined by the manual Edman degradation (→) and carboxypeptidase digestion (⟝) methods. Cys was determined as Cm-cysteine.

was found at positions 29–32 in the sequence of CLBP from *N. naja atra* and LCBP from *N. naja*. This difference probably arose from the misalignment of the tryptic peptides obtained from the latter two proteins. Reinvestigation of the sequences of CLBP from *N. naja atra* and LCBP from *N. naja* is now in progress.

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