Biotechnology Letters (2005) 27: 1597–1603
DOI 10.1007/s10529-005-2514-2

Synthesis, expression and purification of a type of chlorotoxin-like peptide from the scorpion, *Buthus martensii* Karsch, and its acute toxicity analysis

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Received 17 May 2005; Revisions requested 26 May 2005; Revisions received 2 August 2005; Accepted 15 August 2005

Key words: acute toxicity analysis, *Buthus martensii*, chlorotoxin-like peptide, prokaryotic secretive expression

Abstract

A gene, rBmK Cta, encoding a chlorotoxin-like peptide from the scorpion, *Buthus martensii* Karsch, was synthesized according to the sequence optimized for codon usage in *Escherichia coli* and was expressed in *E. coli* BL21 (DE3) using a pExSecI expression system in which the IgG-binding domain-ZZ of protein A is fused to the *N*-terminal of rBmK CTa. The fusion protein, ZZ-rBmK CTa, was expressed in soluble form (7.8 mg l^{-1}) and was purified to give a single band on SDS-PAGE. The domain-ZZ of fusion protein ZZ-rBmK CTa was removed by cleavage of an Asn–Gly peptide bond with hydroxylamine. The rBmK CTa was separated from the IgG-binding moiety by a second passage through the IgG affinity column. Western blot analysis demonstrated that this protein was rBmK CTa. Acute toxicity assay in mice demonstrated that the rBmK CTa had an LD_{50} value of 4.3 mg kg^{-1}.

Introduction

Scorpion venoms are a particularly rich source of small, mainly neurotoxic proteins or peptides interacting specifically with various ionic channels in excitable membranes (Goudet *et al.* 2002). *Buthus martensii* Karsch (BmK) is a representative species of scorpion in northwestern China, Mongolia and Korea (Ji *et al.* 1999). Its neurotoxins have been widely studied in recent years, including investigations into their physiological and biological functions, pharmaceutical properties and primary and three-dimensional structures (Xiong *et al.* 1997, Feng *et al.* 1999). According to their molecular sizes, these neurotoxins can be divided into long- and short-chain neurotoxins (Jia *et al.* 2000). Short-chain toxins are composed of 30–40 amino acid residues and are mainly cross-linked by three or four disulfide bridges and are mostly active on K^+ or Cl^- channels. The peptides, which are a potent block of small conductance Cl^- channels in excitable cells, are called chlorotoxin. The first chlorotoxin-like peptide gene has been cloned and sequenced from the venom of BmK (Wu *et al.* 2000). This 35-mer peptide is cross-linked by four disulfide bridges and shares 66% of its identity with the sequence of chlorotoxin, an inhibitor of small-conductance Cl^- channels from the scorpion *Leiurus quinquestriatus*, which was purified and characterized by DeBin *et al.* (1993). The exact sub-type of Cl^- channels that is affected by BmK CT remains to be identified (Maertens *et al.* 2000).

In this study, a nucleotide sequence of this chlorotoxin-like peptide was synthesized with a sequence optimized for codon usage in *Escherichia coli* with the expectation that this would result in abundant expression. A simple and

efficient purification protocol was designed to get a soluble and functional form of rBmK CTa, which could be used to study the pharmaceutical function of this neurotoxin. Acute toxicity testing was performed using a modified LD_{50} assay (Meier & Theakston 1986). Using this test, it is possible to obtain an LD_{50} using only 8–10 experimental animals, instead of 30 or more.

Materials and methods

Materials

The pExSecI expression system was constructed by Brünen-Nieweler *et al.* (1994) and kindly provided by Prof. K. Heckmann. The plasmid containing BmK CT gene was kindly provided by Prof. C.W. Chi, Institute of Biochemistry and Cell biology in the Chinese Academy of Science. IgG-Sepharose 6 Fast Flow was purchased from Pharmacia biotech (Sweden).

Synthesis of BmK CT cDNA sequence according to the partial genetic codes of E. coli

The BmK CT gene has been isolated previously (Zeng *et al.* 2000) and its nucleotide sequence has been submitted to GenBank database with the accession number AF 135821. In order to get high-level expression in *E. coli*, the BmK CT gene sequence was modified, in which 24 residues (shown in boxes) were replaced according to the codon usage of *E. coli*:

5′-ATG TGC GGT CCG TGC TTC ACT ACT GAC GCT AAC ATG GCT AGA AAA TGC AGA GAA TGC TGT GGT GGT ATC GGT AAA TGC TTC GGT CCG CAG TGC CTG TGC AACCGT ATC TGA –3′

Design of primers and synthesis of recombinant BmK CT artifact (rBmK CTa)

According to the mutated cDNA sequence of BmK CT, four primers was designed to synthesize rBmK CTa:

Primer 1: 5′-ATGTGCGGTCCGTGCTTCACTAC-TGACGCTAACATGGCTAGA-3′
Primer 2: 5′-ATGCTGTGGTGGTATCGGTAA-ATGCTTCGGTCCGCAGTGC-3′
Primer 3: 5′-ACCACAGCATTCTCTGCATTTT-CTAGCCATGTTAGCGTCA-3′
Primer 4: 5′-TCAGATACGGTTGCACAGGCA-CTGCGGACC-3′

The rBmK CTa gene was obtained by using a PCR method. The PCR product was subcloned into a pGEM-T Easy vector and this cDNA sequence was determined by TaKaRa Biotechnology Company.

Construction of expression vector pExSecI-rBmK CTa and expression of ZZ-rBmK CTa in E. coli

The gene, rBmK CTa, was subcloned into an expression vector, pExSecI, using PCR. Corresponding to the coding region of the cDNA sequence, a pair of primers, rBmK CTa (hydroxylamine-*Eco*RI): 5′-AGC GAATTC G **AAT GGG** ATG TGC GGT CCG TGC-3′ and rBmK CTa (*Bam*HI): 5′-GGG GAT CCT CAG ATA CGG TTG CAC AG-3′ with *Eco*RI and *Bam*HI restriction sites were designed. The sequence of rBmK CTa (hydroxylamine-*Eco*RI) coding for Asn–Gly is written in bold letters. The Asn–Gly motif allows for removal of the domain-ZZ of the fusion protein ZZ-rBmK CTa by cleavage with hydroxylamine. The PCR product and plasmid pExSecI were digested with *Eco*RI and *Bam*HI, respectively, and the ligation reactions were performed for obtaining the expression recombinant plasmids pExSecI-rBmK CTa (Figure 1). The plasmids were then transformed into *E. coli* BL21 (DE3) by the heat-shock method. The induction of expression was carried out at 16 °C for about 10 h with 0.4 mM IPTG.

Purification, cleavage of fusion protein and Western blot analysis

Cells were harvested by centrifugation at $8000\times g$ for 10 min and the supernatant, where the fusion protein was secreted, was retained. As shown in Figure 1, the IgG binding domain-ZZ of Protein A is fused to the *N*-terminal of rBmK CTa. Subsequently, the supernatant was applied to 10 ml IgG-Sepharose 6 Fast Flow reagent which was previously equilibrated with Tris/saline/Tween 20 buffer (pH 7.6), it was then blended in the rocking machine at 4 °C for about 1 h to make the ZZ-rBmK CTa attached to the IgG-Sepharose.

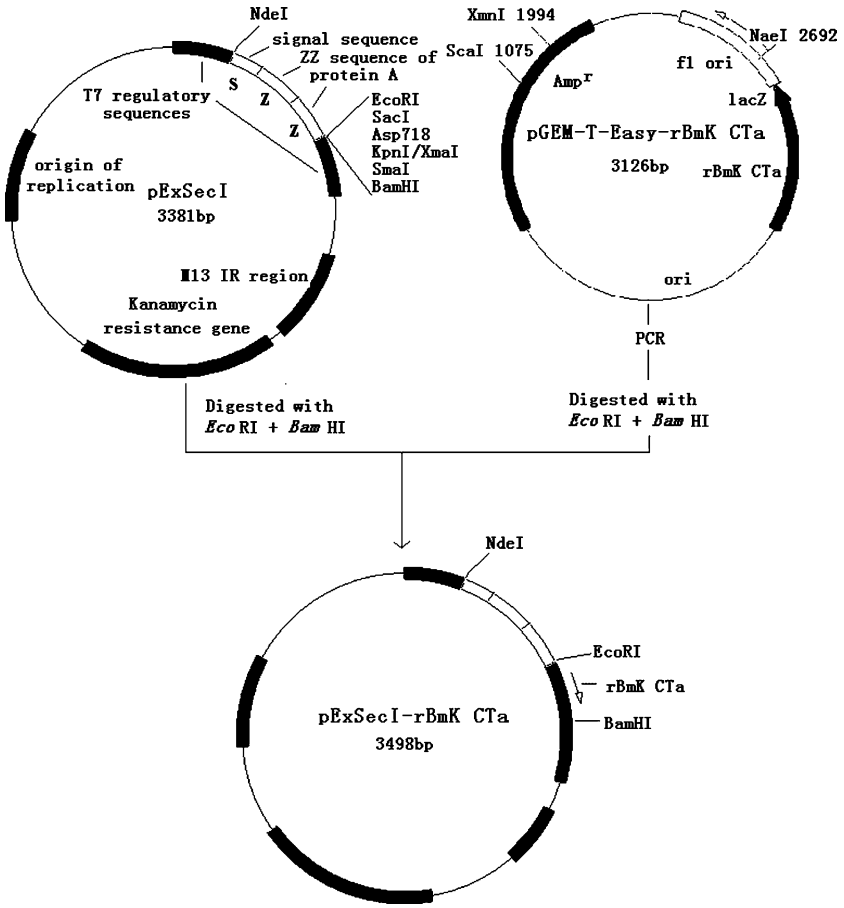

Fig. 1. Construction of expression plasmid pExSecI-rBmK Cta. The physical and genetic maps of pExSecI are shown in this figure. Kanamycin resistance gene, T7 regulatory sequences, signal sequence and ZZ sequence of protein A, origin of replication, M13 IR region and multiple cloning site are represented. The rBmK CTa gene was fused with S-ZZ via the *Eco*RI/*Bam*HI linker. To omit the rBmK CTa leader sequence, rBmK CTa was amplified with two primers, one of which contains the codons for Asn–Gly and an *Eco*RI site. The Asn–Gly motif allows cleavage of the corresponding fusion protein with hydroxylamine.

After washing with 20 ml 5 mM ammonium acetate buffer (pH 5.0), the IgG-Sepharose was eluted with 20 ml 0.5 mM acetic acid (pH 3.4).

The solution of 2 M hydroxylamine hydrochloride and 0.2 M Tris base was titrated to pH 9.0 at 45 °C with NaOH. The protein was dissolved in this solution to give 5 mg ml^{-1}. After incubation at 45 °C for 4 h, the reaction was terminated by lowering the temperature to room temperature and by acidifying with HCl to 8.0. The buffer was exchanged by affinity chromatography (Moks *et al.* 1987). After cleavage, the target protein could be separated from the IgG-binding moiety by a second passage through the IgG affinity column.

The Western blot was probed with rabbit anti-scorpion neurotoxin polyclonal antibody as the first antibody as prepared by the Institute of Zoology in the Chinese Academy of Science. The peroxidase-conjugated goat anti-rabbit IgG (H + L) (ZhongShan Biotechnology Co., Ltd., China) was used as the second antibody. Bound antibodies were detected with the DAB Kit according to the manufacturer's instructions (ZhongShan Biotechnology Co., Ltd., China).

Approximate LD_{50} determinations of rBmK CTa using experimental mice

A method was described for the assessment of lethal toxicity of venoms using a modified LD_{50} assay (Meier *et al.* 1986). Nine 12–14 g male white mice were used for each test. Survival times after injection of different doses of rBmK CTa in mice were recorded to the nearest 5 s.

Results and discussion

Construction of pGEM-T Easy-rBmK CTa

rBmK CTa gene was synthesized and cloned into pGEM-T Easy vector successfully (Figure 2A). The DNA sequence determination confirmed that rBmK CTa was synthesized correctly.

Construction of expression plasmid pExSecI-rBmK CTa

According to the construction of expression plasmid pExSecI-rBmK CTa (Figure 1), pExSecI-rBmK CTa was digested with *Eco*RI and *Bam*HI. The agarose gel-electrophoresis analysis (Figure 2B) and DNA sequence determination confirmed the correct fusion of linker and the target gene in the vector pExSecI.

Expression, purification, cleavage and Western blot analysis of rBmK CTa

The induction of expression was carried out at 16 °C for about 10 h with 0.4 mM IPTG. SDS-PAGE analysis showed that the rBmK CTa was expressed in soluble form. The result of integral analysis (GeneTools software, U.S.A.) showed that the quantity of this peptide reached 19.9% in the total expressed proteins of *E. coli* BL21 (DE3)/ pExSecI-rBmK CTa (Figure 3A, lanes 1 and 2), while the expression level of the corresponding peptide in the stain containing unmodified BmK CT gene (*E. coli* BL21 (DE3)/pExSecI-BmK CT) only accounted for 9.1% (Figure 3A, lane 3). The expressed ZZ-rBmK CTa was harvested and initially purified by the IgG-Sepharose 6 Fast Flow chromatography. 15% SDS-PAGE analysis showed a band with the molecular weight of 18 kDa (Figure 3A, lane 4), corresponding to the calculated molecular weight of ZZ-BmK CTa. Following the above purification scheme, about 7.8 mg ZZ-rBmKa CT was obtained from 1-l Luria-Bertani (LB) culture medium. This fusion protein was cleaved completely with hydroxylamine at 45 °C for 4 h (Figure 3B, lane 3). After cleavage, a band of about 13 kDa (Figure 3B, lane 2) was separated from ZZ protein by a second passage through the IgG-Sepharose 6 Fast Flow chromatography. The yield of affinity-purified protein was 1.4 mg l^{-1} of culture, estimated by the Bradford method. Subsequently, Western blot analysis showed the anti-scorpion neurotoxin polyclonal

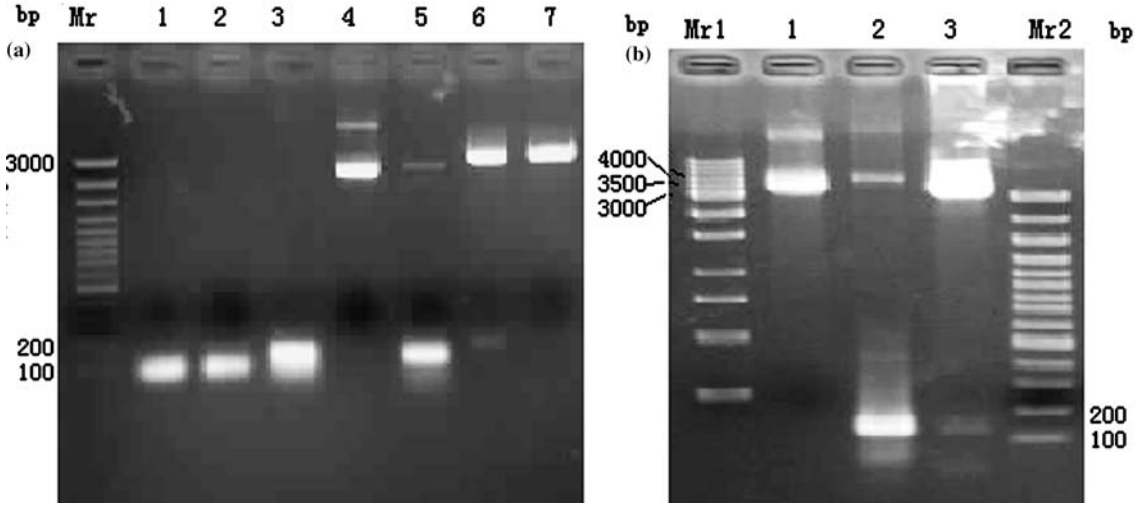

Fig. 2. (a) Synthesis of rBmK CTa and cloning into pGEM-T Easy vector. Mr: Gene Ruler^{TM} 100bp DNA Ladder plus (MBI Fementas); lanes 1,2: Synthesis of the former and back half of rBmKCTa, respectively; lane 3: Synthesis of rBmK CTa by using former and back half of rBmK CTa; lane 4: recombinant plasmid of pGEM-T Easy-rBmK CTa; lane 5: PCR production by primer 1 and primer 4; lane 6: recombinant plasmid pGEM-T Easy-rBmK CTa digested by *Eco*RI; lane 7: recombinant plasmid pGEM-T Easy-raBmK CTa digested by *Xho*I. (b) Analysis of the recombinant pExSecI-rBmK Cta. Mr1: 1 kb DNA Ladder (MBI Fementas); lane 1: recombinant plasmid pExSecI-rBmK CTa; lane 2: PCR product ; lane 3: recombinant plasmid pExSecI-rBmK CTa digested by *Eco*RI and *Bam*HI; Mr2: Gene Ruler^{TM} 100 bp DNA Ladder plus (MBI Fementas).

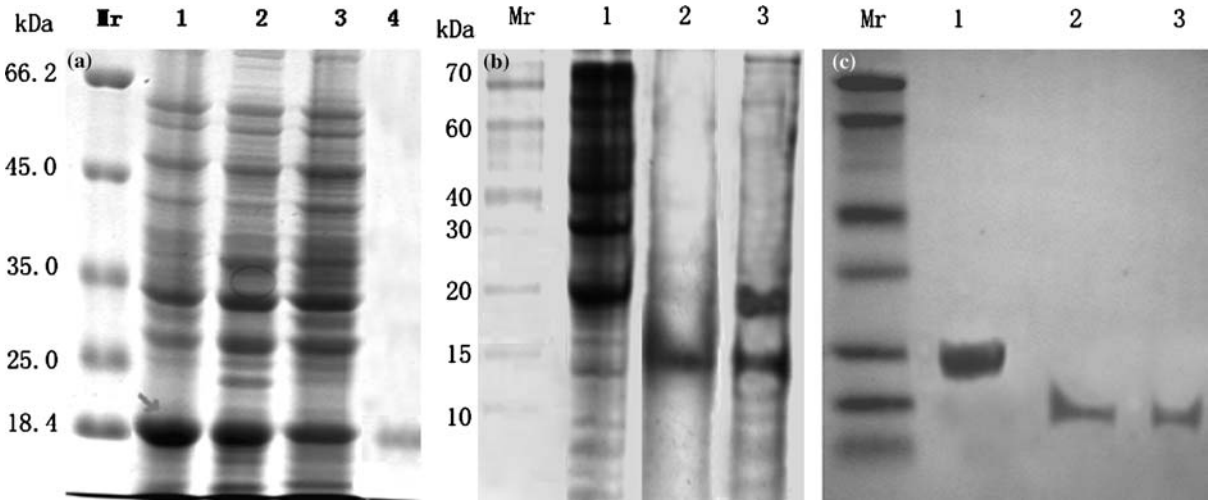

Fig. 3. (a) Expression and purification of the ZZ-rBmK CTa and ZZ-BmK CT protein produced in *E.coli* BL21 (DE3). Mr: Protein molecular weight marker (MBI Fementas); lanes 1,2: the total expressed proteins of two different clones of *E. coli* BL21 (DE3)/pExSecI-rBmK CTa at 4 h. The arrow showed the expression product ZZ-rBmK CTa, The results of integral analysis (GeneTools software, U.S.A.) showed that the quantity of this peptide reached to 19.9% in the total expressed proteins; lane 3: the total expressed proteins of *E. coli* BL21 (DE3) /pExSecI-BmK CT at 4 h, the quantity of this peptide only accounted for 9.1% in the total expressed proteins; lane 4: the purified fusion protein by IgG-sepharose 6 Fast Flow system. (b) Tricine-SDS-PAGE electrophoresis analysis of the purified and cleavaged fusion protein. Mr: Benchmark pre-stained protein ladder (Gibco BRL, U.S.A.) lane 1: total cell extracted proteins which were inducted with IPTG for 4 h; lane 3: cleavaged fusion protein by hydroxylamine; lane 2: purified rBmK CTa by a second passage through the IgG affinity column. (c) Western blot analysis. Lanes 1–4: the same as in Figure 3(B).

antibody reacted specifically with this affinity-purified protein (Figure 3C). The result suggested that the band of 13 kDa could be the tetramer of the recombinant rBmK CTa.

Many attempts at the expression of scorpion neurotoxins in various systems have been reported, including those of *E. coli* (Zilberberg *et al.* 1996, Pang *et al.* 1992), yeast (Feng *et al.* 1999, Zilberberg *et al.* 1996, Martin-Eauclaire *et al.* 1994), insect cells and mammalian cells (Bougis *et al.* 1989, Carbonell *et al.* 1998, Dee *et al.* 1990). Most of them were not very successful because of either the low level of production or the production of a nonfunctional protein. Recently we expressed the gene encoding BmK Mm2 using the pExSecI expression system and got the fusion product ZZ-BmK Mm2 at 1.6 mg l^{-1} (Fu *et al.* 2004). In this study, we obtained the soluble form of a chlorotoxin-like peptide, rBmK CTa, using this pExSecI expression system. Because of the relatively protein-free property of the LB medium, it is easier for us to design this simple and efficient purification protocol as described. The expression level of the fusion protein ZZ-rBmK CTa reached to 7.8 mg l^{-1} by using the sequence optimized for codon usage in *E. coli*. After cleavage with hydroxylamine about 1.4 mg l^{-1} rBmK CTa was obtained and provided the valuable material for further study.

Acute toxicity analysis

The results of observed data, including the calculated dose versus survival time (D/T), for each animal is shown in Table 1. According to this calculation method, the approximate LD_{50} was obtained (Table 2). Acute toxicity assay using nine mice demonstrated that the rBmK CTa had toxicity against animals with an LD_{50} value of 4.3 mg kg^{-1}.

The main advantage of the method is that it permits the use of far fewer experimental animals. As classical LD_{50} experiments, owing to their high level of variability, are far from satisfactory, a change to the approximate method of LD_{50} assays is suggested for scientific, economic and ethical reasons.

Table 1. Survival times observed after injection of different doses of rBmK CTa in mice.*

Animal no.	Weight (±0.2 g)	Dose (D) ($\mu g\ g^{-1}$)	Survival time (T) (±5 s)	Dose/survival Time (D/T)
Control	14	0	$+\infty$	$-\infty$
1	12	5	12 min 03 s	0.42
2	12	6	19 min 15 s	0.31
3	12	7	20 min 17 s	0.35
4	13	8	18 min 52 s	0.43
5	12	10	16 min 17 s	0.62
6	13	12	12 min 39 s	0.97
7	12	15	16 min 04 s	0.94
8	13	20	09 min 39 s	2.13

*Nine 12–14 g male white mice (Kunming strain) were used for this test. rBmK CTa was dissolved in phosphate-buffered saline (PBS) solution to give 0.5, 0.6, 0.7, 0.8, 1.0, 1.2, 1.5 and 2.0 $\mu g\ \mu l^{-1}$, which were then injected into the belly cavity to stated corresponding dose according to the weight of each mouse. Survival times (time between injection and death) were recorded to the nearest 5 s. The calculated dose versus survival time (D/T) for each animal is shown in this table.

Conclusions

We have established an efficient system for expression and purification of a chlorotoxin-like peptide, rBmK CTa, from BmK. Mice were used to test the biological activity of the expressed rBmK CTa. The result of the acute toxicity showed that the expressed rBmK CTa was active and the LD_{50} was 4.3 mg kg^{-1}. The availability of this chlorotoxin-like peptide should be useful to study the mechanism of its binding and action on chloride channel selectively.

Acknowledgments

We thank Dr Michael Borenstein, Associate Dean of Temple University School of Pharmacy, and Dr YuZhu Xue, Laboratory Head, US MPK, Sanofi-Aventis Pharmaceuticals, for reading the manuscript and helping us with the English. The project was supported by the National Natural Science Foundation of China (No. 30270204), Natural Science Foundation of Shanxi (19981084) and Scholar Foundation of Shanxi Province. We thank Prof. Yin and Prof. Yang from Shanxi Medical University for their assistance.

Table 2. Approximate LD_{50} determinations of rBmK Cta.*

Construction	Calculation step	Example
Number of animals	A	8
Sum values		
S_x	B	6.17
S_x^2	C	7.32
S_y	D	83.00
S_y^2	E	1043.00
S_{xy}	F	84.39
$S_{(x-x)}^2 = S_{xx}$	G = C−(B² : A)	2.56
$S_{(x-x)(y-y)} = S_{xy}$	H = F−(BD : A)	20.38
$S_{(y-y)}^2 = S_{yy}$	J = E−(D² : A)	181.88
$S_{xy}^2 : S_{xx}$	K = H² : G	162.24
$S_{yy}-S_{xy}^2 : S_{xx}$	L = J−K	19.64
Means		
$x = S_x/n$	M = B : A	0.77
$y = S_y/n$	N = D : A	10.38
Regression coefficient (slope)		
$a = S_{xy} : S_{xx}$	P = H : G	7.96
LD_{50}		
$b = y-ax$	Q = N−MP	4.25
Standard deviation of a		
$SD_a = (S_{yy}-S_{xy}^2/S_{xx})/(n-2)$	R = ((L : (A−2)) : G)$^{1/2}$	1.13
Standard deviation of b		
$SD_b = (S_{yy}-S_{xy}^2/S_{xx})(1/n + x/S_{xx})/(n-2)$	S = ((R²C) : A)$^{1/2}$	1.08

x = dose/survival time (D/T); y = dose (D).
*The statistical principle of this calculation scheme was explained logically by Meier and Theakston (1986).

References

Bougis PE, Rochat H, Smith LA (1989) Precursors of *Androctonus australis* scorpion neurotoxins: structures of precursors, processing outcomes, and expression of a functional toxin II. *J. Biol. Chem.* **264**: 19259–19265.

Brünen-Nieweler C, Meyer F, Heckmann K (1994) Expression of the pheromone 3-encoding gene of *Euplotes octocarinatus* using a novel bacterial secretion vector. *Gene* **150**: 187–192.

Carbonell LF, Hodge MR, Tomalski MD, Miller LK (1998) Synthesis of a gene coding for an insect-specific scorpion neurotoxin and attempts to express it using baculovirus vectors. *Gene* **73**: 409–418.

Dee AR, Belagaje M, Ward K, Chio E, Lai MHT (1990) Expression and secretion of a functional scorpion insecticidal toxin in cultured mouse cells. *Bio/Technology* **8**: 339–342.

DeBin JA, Maggio JE, Strichartz GR (1993) Purification and characterization of chlorotoxin, a chloride channel ligand from the venom of the scorpion. *Am. J. Physiol.* **264**: 361–369.

Feng S, Xiong YM, Zhu RH, Ling MH, Chi CW, Wang DC (1999) Expression and purification of the BmK M1 neurotoxin from the scorpion *Buthus martensii* Karsch. *Protein Expres. Purifi.* **17**: 358–365.

Fu YJ, Chai BF, Wang W, Zhi H, Yin LT, Liang AH (2004) Expression and purification of the BmK Mm2 neurotoxin from the scorpion *Buthus martensii* Karsch and its biological activity test. *Protein Expres. Purif.* **38**: 45–50.

Goudet C, Chi CW, Tytagt J (2002) An overview of toxins and genes from the venom of the Asian scorpion *Buthus martensii* Karsch. *Toxicon* **40**: 1239–1258.

Ji YH, Li YJ, Zhang JW, Song BL, Toshio Y, Tohru M, Minoru H, Noboru Y (1999) Covalent structures of BmK AS and BmK AS−1, two novel bioactive polypeptides purified from Chinese scorpion *Buthus martrnsii* Karsch. *Toxicon* **37**: 519–536.

Jia LY, Xie HF, Ji YH (2000) Characterization of four distinct monoclonal antibodies specific to BmK AS-1, a novel scorpion bioactive polypeptide. *Toxicon* **38**: 605–617.

Maertens C, Wei L, Tytgat J, Droogmans G, Nilius B (2000) Chlorotoxin does not inhibit volume-regulated, calcium-activated and cyclic AMP-activated chloride channels. *Br. J. Pharmacol.* **129**: 791–801.

Martin-Eauclaire MF, Sogaard M, Ramos C, Cestele S, Bougis PE, Svensson B (1994) Production of active, insect-specific scorpion neurotoxin in yeast. *Eur. J. Biochem.* **233**: 637–645.

Meier J, Theakston RDG (1986) Approximate LD50 determinations of snake venoms using eight to ten experimental animals. *Toxicon* **24**: 395–401.

Moks T, Abrahmsén L, Holmgren E, Bilich M, Olsson A, Uhlén M, Pohl G, Sterky C, Hutberg H, Josephson S, Holmgern A, Jörnvall H, Nilsson B (1987) Expression of human insulin-like growth factor I in bacteria: use of optimized gene fusion vectors to facilitate protein purification. *Biochemistry* **26**: 5239–5244.

Pang SZ, Oberhaus SM, Rasmussen JL, Knipple DC, Bloomquist JF, Dean DH, Bowman KD, Sanford JC (1992) Expression of a gene encoding a scorpion insectotoxin peptide in yeast, bacteria and plants. *Gene* **116**: 165–172.

Wu JJ, Li D, Lan ZD, Chi CW (2000) The gene cloning and sequencing of Bm-12, a Chlorotoxin-like peptide from the scorpion *Buthus martensii* Karsch. *Toxicon* **38**: 661–668.

Xiong YM, Ling MH, Wang DC, Chi CW (1997) The cDNA and genomic DNA sequences of a mammalian neurotoxin from the scorpion *Buthus martensii* Karsch. *Toxicon* **35**: 1025–1031.

Zeng XC, Li WX, Zhu SY, Peng F, Zhu ZH, Wu KL, Yang FH (2000) Cloning and characterization of a cDNA sequence encoding the precursor of a chlorotoxin-like peptide from the Chinese scorpion *Buthus martensii* Karsch. *Toxicon* **38**: 1009–1014.

Zilberberg N, Gordon D, Pelhate M, Adams ME, Norris TM, Zlotkin E, Gurevitz M (1996) Functional expression and genetic alteration of an alpha scorpion neurotoxin. *Biochemistry* **35**: 10215–10222.