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A new insect neurotoxin AngP1 with analgesic effect from the scorpion *Buthus martensii* Karsch: purification and characterization

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Dates:
Received 12 October 2000
Revised 14 November 2000
Accepted 4 February 2001

To cite this article:
Guan, R.-J., Wang, M., Wang, D. & Wang D.-C. A new insect neurotoxin AngP1 with analgesic effect from the scorpion *Buthus martensii* Karsch: purification and characterization.
J. Peptide Res. 2001, **58,** 27–35.

ISSN 1397–002X

Key words: analgesic effect; characterization; purification; scorpion toxin

Abstract: An insect toxin named BmK AngP1 was purified from the venom of the scorpion *Buthus martensii* Karsch (BmK). It also shows an evident analgesic effect on mice, but is interestingly devoid of mammalian toxicity. Bioassay showed that the CPU value of AngP1 was 0.01 μg/body (≈ 30 mg) for the excitatory insect toxicity and 43.0% inhibition efficiency for analgesia at a dose of 5 mg/kg. However, even at the dosage of 10 mg/kg no detectable toxicity on mice could be found. The isoelectric point (pI) value for AngP1 was 4.0, and its molecular mass analyzed by MALDI-TOF MS was 8141.0. The first 15 N-terminal residues of AngP1 were determined by Edman degradation and showed high similarity to that of other excitatory scorpion insect toxins. The circular dichroism spectroscopy measured on a JASCO J-720 system showed that there were 10.4% α-helix, 46.2% β-strand and 14.1% turn structure in this peptide. Under two conditions single crystals of AngP1 were obtained.

Abbreviations: BmK, *Buthus martensii* Karsch; CD, circular dichroism; CPU, contraction paralysis unit; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; pI, isoelectric point; PTH, phenylthiohydantoin.

Scorpion venoms contain a number of peptide neurotoxins active specifically on mammals, insects or crustaceans. They belong to a clearly defined family of peptides that are composed of 60–76 amino acid residues with four disulfide bonds (1–4), and are able to bind to sodium channels impairing depolarization of the action potential in nerve and muscle, resulting in neurotoxicity (5,6). In humans, many

scorpion toxins, e.g. those from the species *Centruroides noxius* (7), *Leiurus quinquestriatus quinquestriatus* (8) and *Tityus serrulatus* (9), can be lethal to an envenomed subject and so cause serious medical problems in Africa and South America.

The scorpion *Buthus martensii* Karsch (BmK) is a widely distributed species in China. Interestingly, its venom is not only much less toxic, never causing the death of an envenomed person, but on the contrary, is used in disease prevention and therapy. In China, many people believe that the scorpion BmK is healthy and one can find them as expensive dishes in restaurants. In fact, scorpion BmK has been one of the indispensable materials used in Chinese traditional medicine for thousands of years. In the first authoritative Chinese pharmacopoeia *Compendium of Material Medica* edited by Li Shi-Zhen in 1578, the pharmaceutical properties and curative effects of the scorpion were described in detail. Whole scorpion, scorpion tails or extract are effective in treating some neural diseases such as apoplexy, epilepsy, facial paralysis and hemiplegia, etc., and are also used to soothe the nerves and relieve pain caused by meningitis, cerebral palsy, rheumatism and so on. Although the pharmacological mechanism remains to be clarified, some active factors from BmK venom have been characterized. For example, a peptide with an antiepilepsy effect has been isolated and partially sequenced (10), the analgesic effect has been confirmed in crude BmK venom (11), and an excitatory insect toxin with an analgesic effect named BmK IT-AP was cloned and sequenced (12). This implies that there may be more active peptides with important clinical applications. In recent experiments we found another active peptide with an analgesic effect that is different from the BmK IT-AP identified previously. Interestingly, this new peptide also shows anti-insect toxicity. Here we report the purification and characterization of this active peptide, BmK AngP1, which shows an evident analgesic effect with simultaneous excitatory insect toxicity, but is devoid of any toxicity on mice even at a high dosages.

Materials and Methods

Sample and chemicals

Venom from scorpion BmK was obtained by electrical stimulation of animals raised in Henan Province, China. All gels used in chromatography and electrophoresis were from Pharmacia. The acetonitrile used in reverse-phase chromatography was gradient grade (Merck). The α-cyano-4-hydroxycinnamic acid used in mass spectroscopy was from Sigma. The reagents used in N-terminal sequencing were from Perkin–Elmer. HEPES used in crystallization was from Sigma. All other chemicals were of analytical grade.

Purification of BmK AngP1

After centrifugation on a Sigma-202 MK centrifuge at 6000 r.p.m. for 30 min, 400 mg of scorpion BmK venom dissolved in 4 mL 0.05 M NH_4HCO_3 for 5–7 days was applied to a Sephadex G-50 column (2.6×150 cm) which had been equilibrated previously with 0.05 M NH_4HCO_3. The column was then eluted with the same solution at a flow rate of 18 mL/h and fractions of 6 mL per tube were collected. Elution was detected by UV280 absorption using a LKB BROMMA UV-detector and recorder. Fractions were pooled and lyophilized.

The fraction containing analgesic activity was dissolved in 5 mL 0.02 M sodium phosphate buffer at pH 6.4, and centrifuged at 6000 r.p.m. for 15 min It was then subjected to a pre-equilibrated Sephadex SP C-25 ion-exchange column (1.6×80 cm) which was first eluted with the same phosphate buffer then with a gradient of 0.1–0.8 M sodium chloride at a flow rate of 24 mL/h to fractionate the various toxins. The peaks were collected and concentrated. Desalting was completed on a Sephadex G-25 column (1.6×80 cm) eluted with 5% acetic acid at a flow rate of 60 mL/h.

Further separation was carried out by preparative isoelectrofocusing electrophoresis on LKB 2117 Multiphor system with Ultrodex granule gel in the ampholytes pH range 3.5–9.5. The sample focused in the gel was eluted and recovered with 5% acetic acid. After concentration, the ampholytes were removed on a Sephadex G-25 column as described above.

Bioassay

Determination of analgesic activity

The analgesic activities of the resulting peptides were assessed with a mouse-twisting model (13) used widely in painkiller screening, according to the 'Guidance to Drug Evaluation in Pre-clinical Test and Research' (14). ICR mice (male, SPF level, mass 18–20 g) from Beijing Experimental Animal Center were used in the test. Mice were injected intraperitoneally with 0.2 mL of 1% acetic acid solution to induce extensive and long-lasting aches in their internal organs. In response to the aches mice twist their bodies, so the twisting action reflects the intensity of the aches. If the

twisting action can be eased by the injection of sample into the mouse, it means that this sample has an analgesic effect. To perform the bioassay, 0.2 mL toxin solution at various concentrations was injected intravenously into the tail of the mouse, using 0.9% NaCl solution as a control. Twenty minutes later, 0.2 mL 1% acetic acid solution was injected intraperitoneally. Five minutes later the mouse twisting action was counted for 10 min. At each dose including the control, a group of 10 mice was tested. The result was analyzed statistically using *t*-test.

Toxicity in insects

The anti-insect toxicity activity of the final obtained sample was tested using housefly larvae as described previously (15). After culture for 5–6 days, fly larvae of body mass ≈ 30 mg were selected for the toxicity test. The sample dissolved in 0.9% NaCl solution was injected into the lower part of the abdomen at different doses. A group of 10 larvae was injected with the same sample amount. The reaction of the larvae was observed for at least 5 min The amount of sample injected and the reaction of the larvae after injection were recorded.

Toxicity in mammals

In order to test whether the analgesic effect of BmK AngP1 is relevant with the syndrome of the mammal-directed toxicity, the toxicity of AngP1 in mammals was tested with ICR mice as described above. Using 0.9% NaCl as the control and groups of 10 mice, various doses of the sample dissolved in 0.9% NaCl were injected into the mice through the tail vein. The reaction and the doses were recorded.

Molecular mass determination

The sensitive and quick matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was selected to analyze the molecular mass of this active peptide. The MALDI MS was measured using a BIFLEX III-MS (Bruker). The matrix used was α-cyano-4-hydroxycinnamic acid, which was dissolved saturated in a 1 : 1 mixture of 1% trifluoroacetic acid and acetonitrile. The mixed solution was used for sample preparation. A sample of 200–300 pmol was used to complete the experiment. The external mass calibration was accomplished by using cytochrome *c* as a peptide standard.

Circular dichroism spectroscopy

The peptide purified was dissolved in 20 mM sodium phosphate buffer at pH 7.0 with a concentration of 1.0 mg/mL. A 30-μL aliquot of the sample solution was applied to a JASCO J-720 system (Japan Spectroscopy Co. Ltd) to perform circular dichroism (CD) measurements. Experiments were carried out in the far UV-range, 200–250 nm, at 298 K. The sample was measured eight times and no significant differences were found. The results were then averaged. Estimation of the secondary structure from the CD spectrum was carried out with the estimation program provided by the company.

N-Terminal amino acid sequencing

The purified sample (1 nmol in double-distilled water) was used directly for N-terminal sequence analysis by automated Edman degradation with an Applied Biosystems 475A Protein Sequencer (USA). Phenylthiohydantoin (PTH) derivatives of amino acids were separated and identified by an on-line PTH analyzer (Model 120A, Applied Biosystems) on a PTH-C_{18} column (5 μm, 220×2.1 mm), eluted with a linear gradient of acetonitrile.

Crystallization

The purified sample was dissolved in 0.001 M hydrochloric acid at a concentration of 20 mg/mL, and the crystallization experiments were carried out using hanging-drop vapor diffusion method in a wide range of precipitants and pH values.

Results

Purification of BmK AngP1

Pure sample was obtained using a successful combination of chromatography and preparative isoelectrofocusing electrophoresis. After being lyophilized to powder, the second fraction eluted from the Sephadex G-50 column (Fig. 1A) which showed analgesic activity was then applied to a Sephadex SP C-25 column. The flow-through fraction was pooled according to its analgesic effect and then desalted on a Sephadex G-25 column. There were two protein peaks from the desalting column (Fig. 1B); BmK AngP1 was purified from the first. The collected sample was then subjected to preparative isoelectrofocusing electrophoreses.

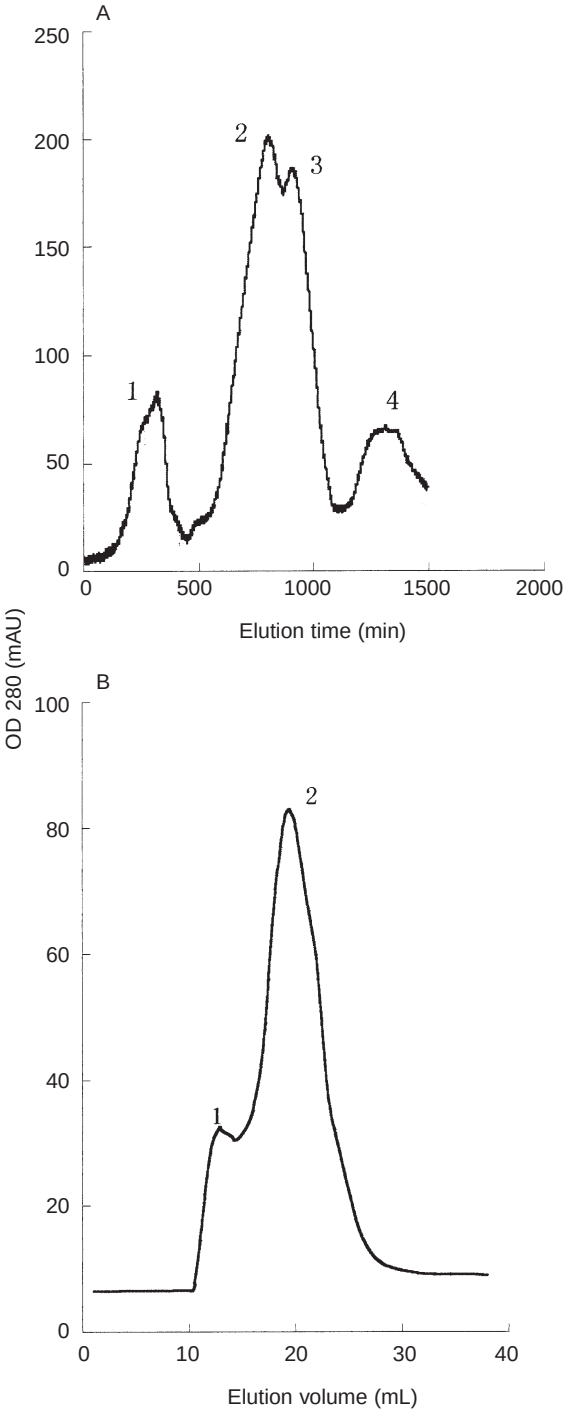

Figure 1. (A) Elution curve of the venom on the Sephadex G-50 column. The second fraction was pooled according to its analgesic activity. (B) Elution curve on the desalting Sephadex G-25 column. AngP1 was purified from the first peak.

The sample focused at pH 4.0 was named BmK AngP1 (Fig. 2.). After disposing of the ampholytes, the final sample of BmK AngP1 was obtained. The analysis with a C_8 reverse-phase column (5 μm, 4.6/250) on a ÄKTA Purifier system (Pharmacia.) showed that it possessed purity >90% (Fig. 3).

Bioassay

Analgesic activity

Tested using the mouse-twisting model, BmK AngP1 showed obvious analgesic bioactivity. The experimental data and data processing are listed in Table 1. The inhibition curve is shown in Fig. 4. Because the *t*-values in Table 1 at each dose were all $>t_{(18)\,0.01}$ (2.878), the *P*-value was <0.01. BmK AngP1 showed 43.0% inhibition efficiency at a dose of 5 μg/g (0.699 in Log form on the horizontal axis in Fig. 4), whereas a pain killer, tranquillizer, composed of 60% aminopyrinum, 30% propylphenazone and 10% barbitalum, used commonly in clinics, showed only 62.4% inhibition activity at a dose of 80 μg/g (shown in a parallel experiment) (12).

Toxicity in insects

The toxicity of the purified AngP1 in insects was tested using housefly larvae. In the experiments, the larvae contracted immediately after injection of the sample, and no flaccid paralysis was observed later, which indicated that AngP1 belonged to the group of excitatory insect toxins. The contraction paralysis unit (CPU), defined as the acute dose that caused half of the recipient larvae immediately contracted in 5 s, value for AngP1 was 0.01 μg/body (≈30 mg).

Toxicity in mammals

The toxicity of AngP1 in mammals was tested using ICR mice. The results showed that no paralytic symptoms could be observed for AngP1 even at a dose of 10 mg/kg, which is 15 times higher than the LD_{50} (the dose that causes the death of half of the mice tested) of a typical mammal-directed toxin, BmK M1, determined using the same procedure (16). This is also the highest dose to induce the detectable mammal-directed toxicity in all BmK toxins identified to date (16). The result indicated that, interestingly, AngP1 was devoid of mammalian neurotoxicity.

Molecular mass

In order to determine the exact molecular mass, BmK AngP1 was analyzed by MALDI-TOF MS on a BIFLEX III-MS. The mass spectrum (Fig. 5) showed that the molecular mass of AngP1 was 8141.0 Da.

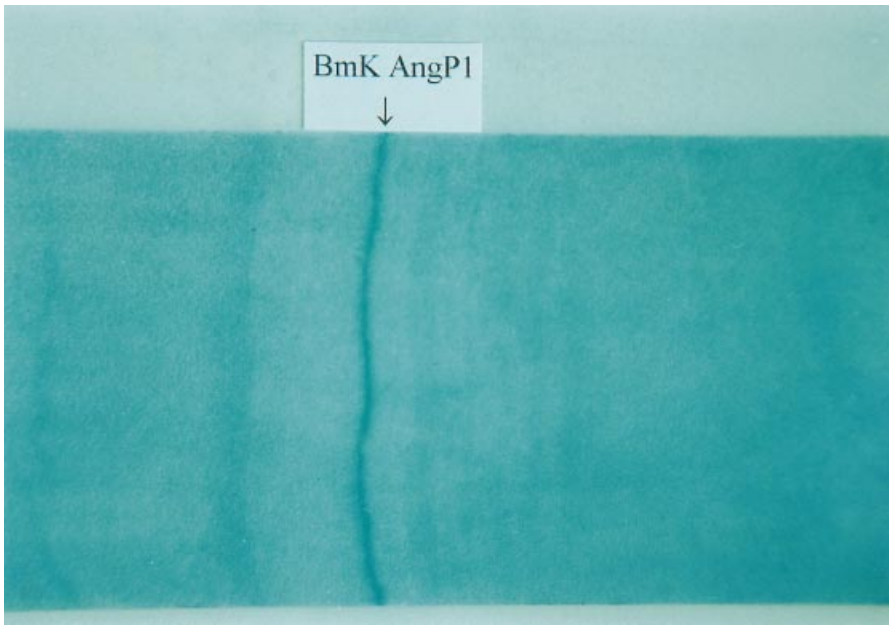

Figure 2. Photograph of the preparative electrophoresis gel, in which BmK AngP1 was focused at the marked position, pH 4.0.

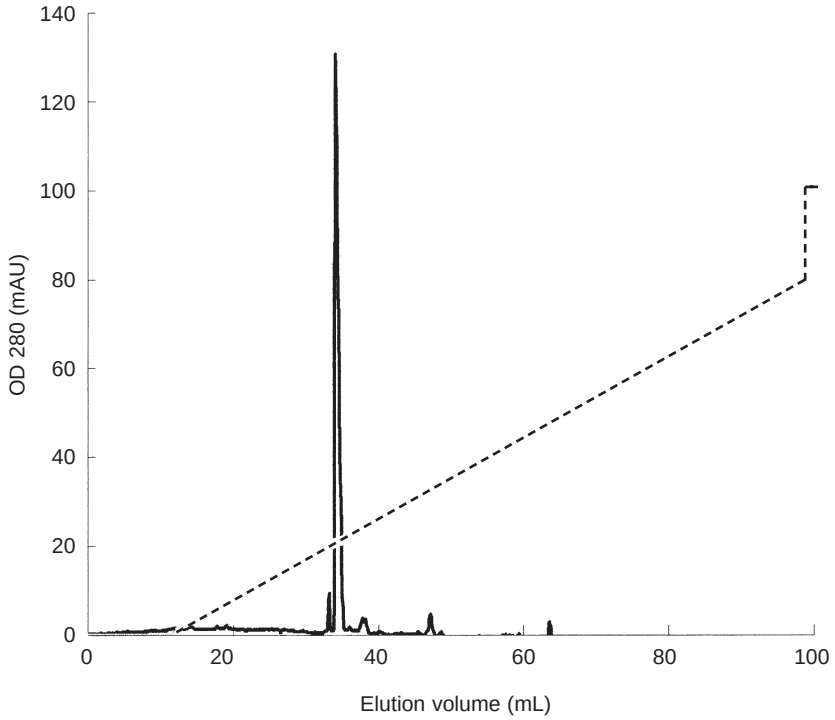

Figure 3. Reverse-phase analysis of BmK AngP1 on a C_8 column (dashed line indicates the gradient of acetonitrile), which showed that BmK AngP1 had a purity >90%.

CD spectra

The secondary structure of BmK AngP1 was detected using CD. Figure 6 shows the CD spectrum of AngP1 in pH 7.0 phosphate buffer at room temperature. The secondary structure estimation (J-700 for Windows Secondary Structure Estimation, Version 1.10.00) from the CD data showed that BmK AngP1 was rich in β-structure (46.2%) with a less amount of helix structure (10.4%) as well as 14.1% turn. The result is basically coincident with that for the NMR structure of an insect toxin, AaH IT (17), which contains 14.3% α-helix, 20% β-structure and 11.4% turn.

N-Terminal amino acid sequence of BmK AngP1

The first 15 N-terminal amino acid residues of BmK AngP1 were definitely determined as KKNGYAVDSSGKVAE.

Table 1. **Statistics of analgesic effect determination for BmK AngP1**

	Dose (mg/kg)	Log (dose)	Twisting count (x)	$\bar{x}$	Inhibition efficiency (%)	S	S_c^2	$S_{(\bar{x}_1-\bar{x}_2)}$	t	p
Control			56, 65, 55, 56, 60, 64, 60, 58, 65, 62	60.1		3.81				
AngP1	1.0	0.0	51, 52, 51, 49, 47, 51, 54, 49, 50, 53	50.7	15.6	2.06	9.38	1.37	6.86	<0.01
	1.5	0.176	45, 43, 47, 49, 44, 45, 47, 45, 47, 44	45.6	24.1	1.84	8.95	1.34	10.84	<0.01
	2.5	0.398	41, 38, 41, 39, 42, 43, 42, 37, 44, 41	40.8	32.1	2.20	9.68	1.39	13.87	<0.01
	3.25	0.512	45, 47, 41, 46, 43, 39, 46, 41, 44, 37	42.9	28.6	3.31	12.74	1.60	10.78	<0.01
	5.0	0.699	32, 29, 30, 31, 33, 31, 34, 32, 31, 35	31.8	47.1	1.81	8.90	1.33	21.22	<0.01

The control was 0.9% NaCl. See the calculation of inhibition efficiency in the legend for Fig. 4. The formulae used in the statistics are listed as follows: (Here $n_1=n_2=10$).

$$\bar{x}=\sum_{i=1}^{n}x_i \qquad S=\sqrt{\frac{\sum(x-\bar{x})^2}{n-1}} \qquad t=\frac{|\bar{x}_1-\bar{x}_2|}{S_{(\bar{x}_1-\bar{x}_2)}} \qquad S_c^2=\frac{(n_1-1)S_1^2+(n_2-1)S_2^2}{(n_1-1)+(n_2-1)} \qquad S_{(\bar{x}_1-\bar{x}_2)}=\sqrt{S_c^2\left(\frac{n_1+n_2}{n_1n_2}\right)}$$

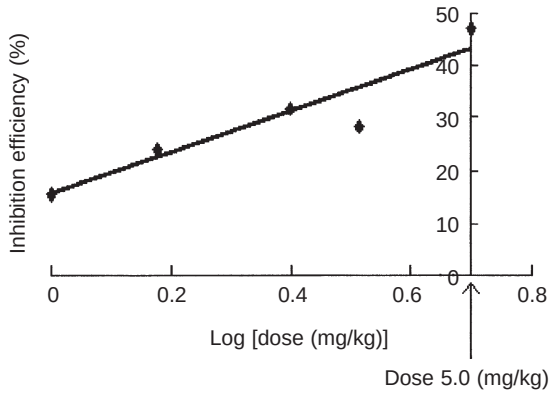

Figure 4. Analgesic activity curves of BmK AngP1. The inhibition efficiency is defined as the ratio of $(N_o–N)/N_o$, in which N_o is the mean twisting times for the control group counted for 10 min, and N is the mean twisting times of the experimental group with different dosage of AngP1. The marked position indicates the 43.0% inhibition efficiency at a dose of 5.0 mg/kg.

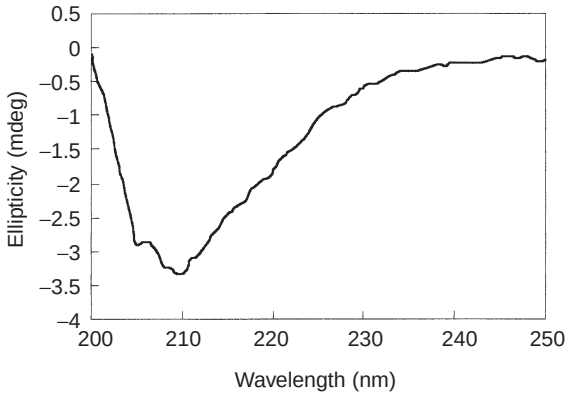

Figure 6. CD spectrum of BmK AngP1 in the UV range 200–250 nm analyzed on a JASCO 720 system at pH 7.0 in a concentration of 1.0 mg/mL at room temperature.

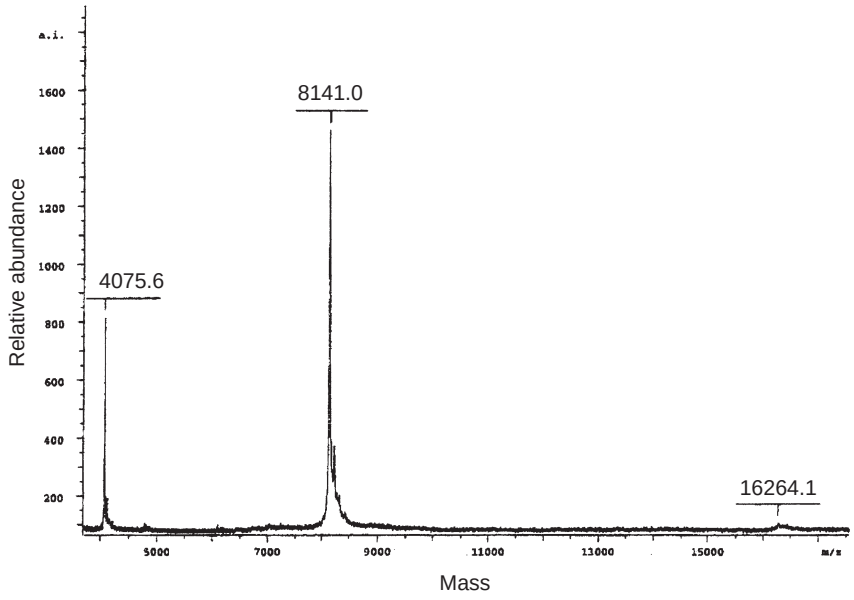

Figure 5. MALDI-TOF mass spectrum of BmK AngP1 determined on BIFLEX III-MS.

	10	20	30	40	50	60	70	77	reference
Bj-xtrIT	KKNGYPLDRN	GKTTECSGVN	AIAPHYCNSE	CTKVYYAESG	YCCWGACYCF	GLEDDKPIGP	MKDITKKYCD	VQII-PS	[18]
BmK IT-AP	KKNGYAVDSS	GKVAECLFNN	-----YCNNE	CTKVYYADKG	YCCLLKCYCF	GLADDKPVLD	IWDSTKNYCD	VQIIDLS	[12]
AaH IT	KKNGYAVDSS	GKAPECLLSN	-----YCNNQ	CTKVHYADKG	YCCLLSCYCF	GLNDDKKVLE	ISDTRKSYCD	TTIIN	[19]
AaH IT1	KKNGYAVDSS	GKAPECLLSN	-----YCNNE	CTKVHYADKG	YCCLLSCYCF	GLNDDKKVLE	ISDTRKSYCD	TTIIN	[20]
AaH IT2	KKNGYAVDSS	GKAPECLLSN	-----YCYNE	CTKVHYADKG	YCCLLSCYCF	GLNDDKKVLE	ISDTRKSYCD	TPIIN	[20]
Lqq IT1	KKNGYAVDSS	GKAPECLLSN	-----YCYNE	CTKVHYADKG	YCCLLSCYCV	GLSDDKKVLE	ISDARKKYCD	FVTIN	[8]
BmK AngP1	KKNGYAVDSS	GKVAE							(This work)

Figure 7. N-Terminal sequence of BmK AngP1 and full sequences of some other excitatory insect-specific toxins.

Comparison of the N-terminal sequence of AngP1 with other excitatory toxins is shown in Fig. 7. Very high homology can be found among the first 15 residues of these peptides.

Crystallization

Using the hanging-drop vapor-diffusion method, after screening and optimizing the precipitants and pH values, single crystals of BmK AngP1 were obtained under the two conditions listed in Table 2. Photographs of the crystals are given in Fig. 8.

***Table 2.* Crystallization conditions for BmK AngP1**

	Condition I	Condition II
Solution A	20 mg AngP1/mL H_2O	20 mg AngP1/mL H_2O
Solution B	4.0 M sodium chloride, 0.05 M HEPES, pH 7.5	0.8 M sodium citrate, 0.05 M HEPES, pH 7.5
Drop	1.0 μL A+1.0 μL B	1.0 μL A+1.0 μL B
Reservoir	600 μL solution B	600 μL solution B
Temperature	295 K	295 K

Discussion

The results definitely show that BmK AngP1 is a novel active peptide with dual bioactivities of an analgesic effect and excitatory insect toxicity. The main data presented here, from chromatographic behavior to molecular characterization, show that AngP1 and BmK IT-AP (12) are distinct. They possess different pI values and molecular mass, as well as various potencies of analgesic effects and insect toxicities.

Interestingly, these two peptides exhibit an obvious analgesic effect on mice but no detectable toxicity to them. Therefore, the analgesic effects of AngP1 and IT-AP can not be ascribed to any mammalian neurotoxicity the peptides may have. Besides, the bioassay showed that, for AngP1, the analgesic effect on mice (15.0% inhibition efficiency at 1.0 mg/kg dose and 43.0% at 5.0 mg/kg dose) is at least 4–5 times weaker than that of IT-AP (53.0% inhibition efficiency at 1.0 mg/kg dose) (12), but the toxicity to insects (CPU: 0.01 μg/body; ≈ 30 mg) is twice as strong as that of IT-AP (CPU: 0.02 μg/body; ≈ 30 mg) (12). That means that the analgesic effects of these peptides are not parallel to the insect toxicity. Thus, these two bioactivities may have distinct structure–function determinants. To understand the molecular basis it is necessary to determine their three-dimensional structures. The successful crystallization of AngP1 has provided a sound basis for further investigation.

Several excitatory insect toxins have been isolated from the venoms of other scorpion species, such as AaH IT1, IT2 from *Androctonus australis* Hector (20), Lqq IT1 from *Leiurus quinquestriatus quinquestriatus* (8), and the expressed one Bj-xtrIT from *Buthotus judaicus* (18). However, with the exception of AngP1 and IT-AP, none has been reported as having an analgesic effect. The sequence alignment (Fig. 7) shows that they are highly homologous. A general question was therefore raised, do all excitatory insect toxins have the analgesic effect? This is open to the further investigation.

Although all analgesic chemicals in common use, such as morphine, heroin, barbitone and aspirin, can soothe the nerves and relieve pain, they also have some side-effects, in particular the narcotic drugs which are addictive. Bearing in mind that BmK scorpions have been used as a traditional medicine for about a 1000 years in China, further exploration might provide a potential medicine for analgesia from the BmK AngPs without the danger of addiction.

Acknowledgements: This work was supported by the National Basic Research Program of the MOST, the National Natural Science Foundation, China-Flanders Grant and the '921' Research Plan of China. We thank Ms Shi Xiang and Mr Liu Wen-She for their helps in bioassay for AngP1.

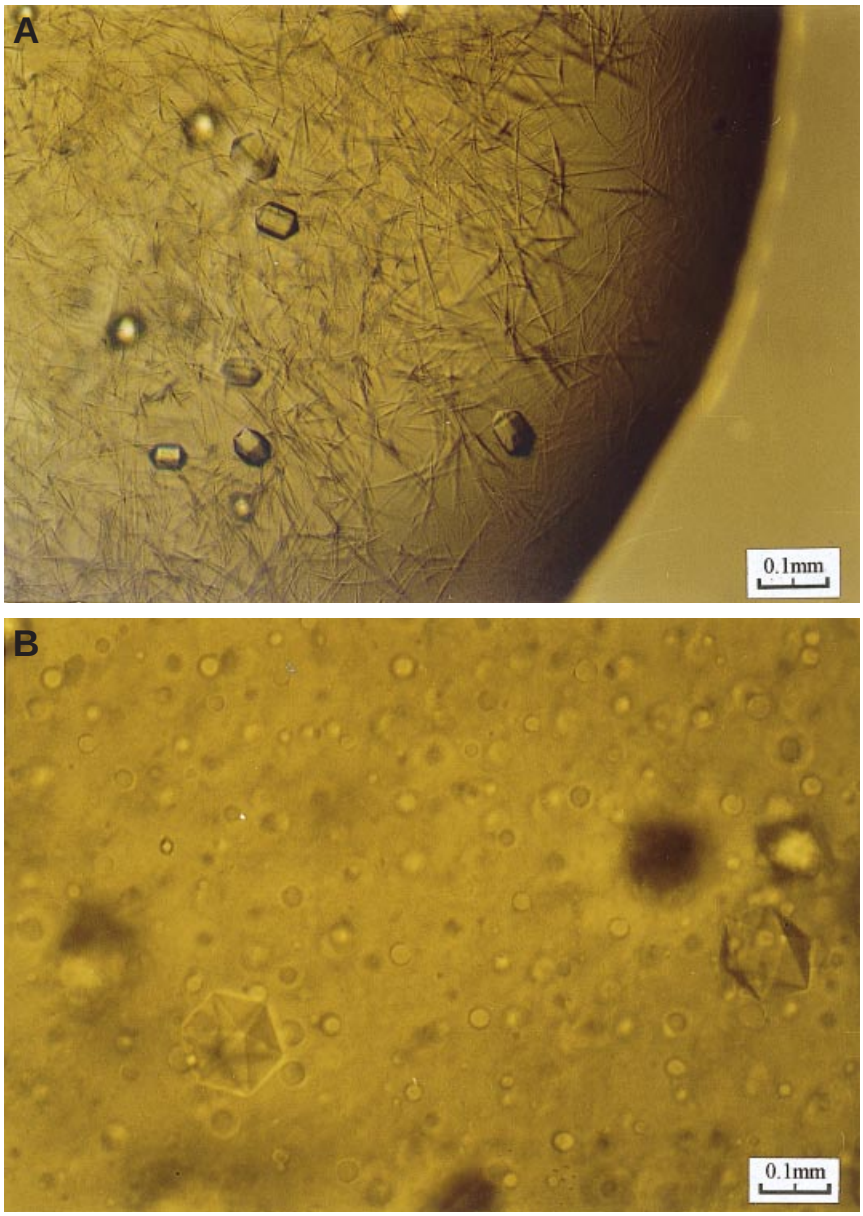

Figure 8. Photographs of AngP1 crystals under (A) condition I and (B) condition II in Table 2.

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