ГЕНОМИКА.
ТРАНСКРИПТОМИКА

PURIFICATION, CHARACTERIZATION AND cDNA CLONING
OF AN ANALGESIC PEPTIDE FROM THE CHINESE SCORPION
Buthus martensii Karsch (BmK AGP-SYPU2)

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In this study an analgesic peptide was purified through five continuous chromatographic steps. The mouse twisting model test was used to identify the target peptides in every separation step. The purified BmK AGP-SYPU2 was further qualified by Reverse Phase-High Performance Liquid Chromatography and High Performance Capillary Electrophoresis. The molecular weight, isoelectric point, and N-terminal sequence of the purified peptide were determined. Based on the N-terminal sequence, the cDNA was cloned by rapid amplification of the cDNA ends from the cDNA pool of scorpion glands. Sequence determination showed that the mature BmK AGP-SYPU2 peptide is composed of 66 amino acid residues, and BmK AGP-SYPU2 is identical to BmK α2 (GenBank Acc. No. AF288608) and BmK αTX11 (GenBank Acc. No. AF155364). We report herein a purification procedure that yields substantial amounts of natural BmK AGP-SYPU2 with high analgesic activity.

The scorpion *Buthus martensii* Karsch (BmK) is a widely distributed species in China, which has been used in traditional Chinese medicine for thousands of years. Whole scorpions, scorpion tails and their extracts have been found to be effective in treating certain neurological diseases, such as apoplexy, epilepsy, facial paralysis, and hemiplegia, in addition to their uses for soothing nerves and relieving pains caused by meningitis, cerebral palsy and rheumatism [1]. After testing scorpion venom on rats, mice and monkeys in five different models, Li et al. [2] reported that this venom did not elicit dependence. Analgesic substances that are commonly used, such as morphine, heroin, and aspirin, can soothe the nerves and relieve pain, but they also have certain side effects, in particular, the addictive nature of narcotic drugs. Therefore, further exploration of BmK might provide a potential medicine for analgesia. In the last few years, an increasing number of analgesic peptides from scorpion has been found, such as BmK IT2 [3]; BmK I1, I4, and I6 [4]; BmK dITAP3 [5]; BmK AS; BmK AS-1 [6]; BmK Ang P1 [7]; BmK AGAP [1]; BmK Ang M1 [8]; and BmK AGP-SYPU1 [9].

In the present study purification, characterization and cDNA cloning of an analgesic peptide from the Chinese scorpion, designated as BmK AGP-SYPU2, are described. This peptide was isolated and purified by ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and gel filtration chromatography (GF). Our studies also demonstrated that BmK AGP-SYPU2 exhibits clear analgesic effects *in vivo* when compared with the pain killer morphine.

EXPERIMENTAL

Materials and Equipment. Scorpion venom collected by electrical stimulation was purchased as a lyophilized product from Qinhuang Dao, He-Bei province, China. The chromatography media, including SP Sepharose Fast Flow, Q Sepharose Fast Flow and Phenyl Sepharose 4 Fast Flow, were purchased from "Amersham Pharmacia Biotech" (Sweden). The columns of Superdex Peptide HR 10/30 and μRPC C2/C18 ST 4.6 × 100 mm were from "Amersham Pharmacia Biotech". Trifluoroacetic acid (TFA) and acetonitrile were purchased from "Merck" (Germany). RevertAidTM M-MuLV reverse transcriptase was obtained from "Fermentas" (Lituva). The pGEM-T Easy T Overhang Vector System was obtained from "Promega". The ÄKTA Purifier 100 chromatography system and gel electrophoresis apparatus were from "Amersham Pharmacia Biotech". The mice used in

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Abbreviations: BmK − *Buthus martensii* Karsch; IEC − Ion exchange chromatography; HIC − Hydrophobic interaction chromatography; GF − Gel filtration chromatography; IEF − Isoelectric focusing; HPCE − High Performance Capillary Electrophoresis.
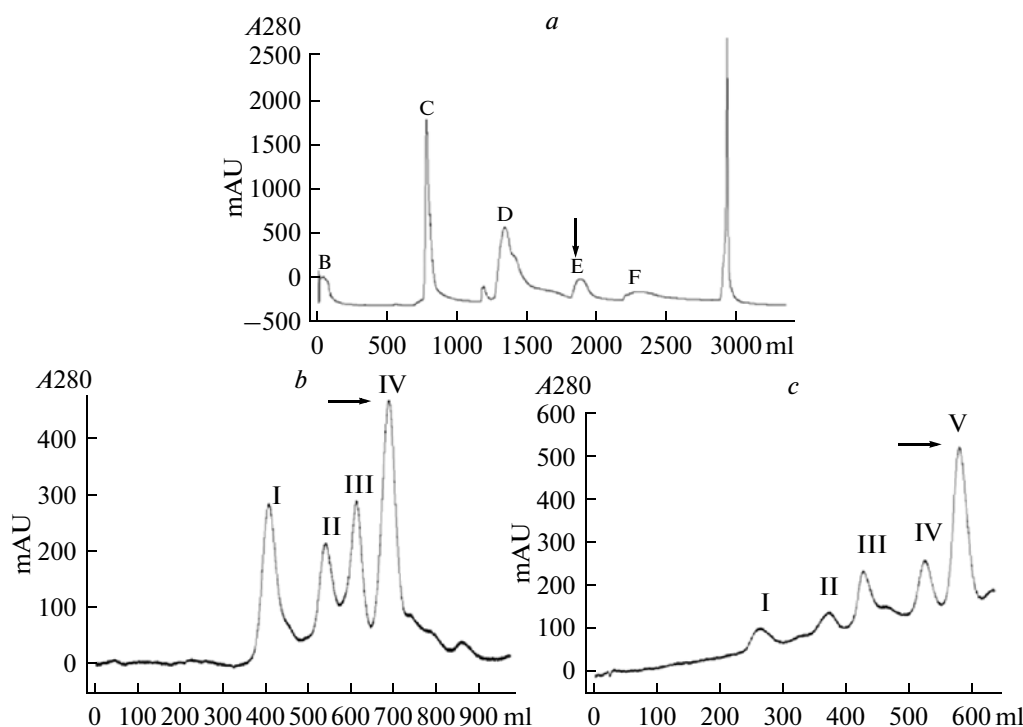*E-mail: Jinghaizhang82@gmail.com

Fig. 1. Separation and purification procedure for the active analgesic peptide from BmK venom. *a* − Crude venom separated by cation exchange chromatography on SP Sepharose Fast Flow. *b* − Second step: further separation by cation exchange chromatography on SP Sepharose Fast Flow. *c* − Third step: separation on Phenyl Sepharose Fast Flow.

the bioactivity assay were Kunming mice from the animal center of Shenyang Pharmaceutical University. Unless otherwise stated, all laboratory reagents were of analytical grade or better.

Preparation of scorpion venom solution. Lyophilized scorpion venom powder (5.0 g) was dissolved in 50 ml of buffer A_1 (50 mM phosphate buffered saline, pH 6.0) and stored at room temperature overnight. After centrifugation at 10000 *g* for 10 min at 4°C, the supernatant was used in subsequent separation and purification procedures.

Cation exchange chromatography: Steps 1 and 2. The first separation step was carried out on a SP Sepharose Fast Flow column (2.6 × 10 cm). The column was equilibrated with buffer A_1, which allowed the binding of the desired solute molecules. The sample solution in buffer A_1 was loaded onto the column at a flow rate of 1.0 ml/min. Elution was carried out at a flow rate of 2.0 ml/min by five steps of increasing ionic strength using 500 ml of each of the following buffers: B_1 (A_1 + 100 mM NaCl), C_1 (A_1 + 200 mM NaCl), D_1 (A_1 + 300 mM NaCl), E_1 (A_1 + 500 mM NaCl), and F_1 (A_1 + 1.0 M NaCl).

The buffer E_1 fraction (fig. 1*a*) contained the analgesic activity and was used for a second separation step on another SP Sepharose Fast Flow column (1.6 × 20 cm). The buffer E_1 fraction was concentrated and desalted

by ultrafiltration, adjusted to pH 4.5, and loaded onto the column, which was equilibrated with buffer A_2 (10 mM Na_2HPO_4 + 5 mM citric acid, pH 4.5). Elution was carried out at a flow rate of 2.0 ml/min with a linear ionic strength gradient of buffer B_2 (10 mM phosphate buffered saline, pH 6.6) to 100% buffer C_2 (B_2 + 350 mM NaCl) using 320 ml of each buffer.

Hydrophobic interaction chromatography. The analgesic fraction from the second SP Sepharose chromatographic step was further purified by HIC on a Phenyl Sepharose 4 Fast Flow column (1.6 × 20 cm). The column was equilibrated with 80 ml of buffer A_3 (20 mM phosphate buffered saline, pH 7.0 + 2.0 M ammonium sulfate). Peak IV from the second SP Sepharose chromatographic step (fig. 1*b*) was loaded onto the column after ammonium sulfate had been added to the fraction at a concentration of 2.0 M, and the pH was adjusted to pH 7.0. Elution was carried out at a flow rate of 2.0 ml/min with a linear descending salt gradient of buffer A_3 to 100% buffer B_3 (20 mM phosphate buffered saline, pH 7.0) using 320 ml of each buffer.

Anion exchange chromatography. The analgesic fraction from the HIC step was further purified by anion exchange chromatography on a Q Sepharose Fast Flow column (1.6 × 20 cm). Peak V from the HIC (fig. 1*c*) was concentrated and desalted by ultrafiltra-
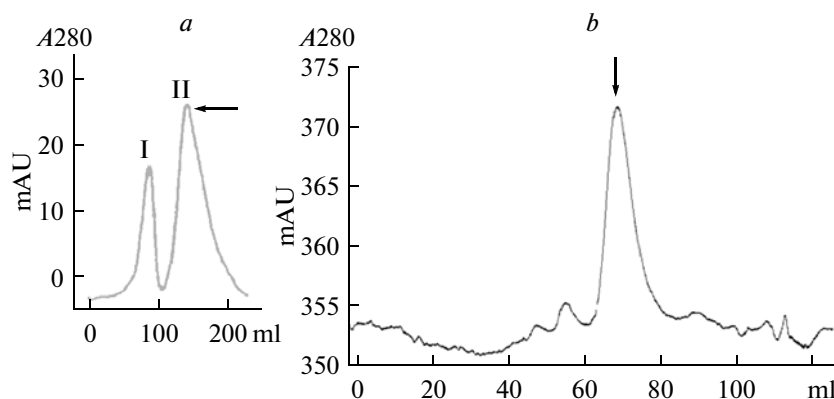
Fig. 2. Separation and purification procedure for the active analgesic peptide from BmK venom. *a* − Fourth step: separation by anion exchange chromatography on Q Sepharose Fast Flow. *b* −Fifth step: separation by gel filtration on Superdex Peptide HR 10/30.

tion, adjusted to pH 11.5, and loaded onto the column, which was equilibrated with buffer A_4 (100 mM Na_3PO_4 + HCl, pH 11.5). Elution was carried out at a flow rate of 2.0 ml/min with a linear ionic strength gradient of buffer B_4 (20 mM Na_3PO_4 + HCl, pH 11.5) to 100% buffer C_4 (B_4 + 1 M NaCl) using 200 ml of each buffer.

Gel filtration chromatography. Based on the molecular weight of known scorpion neurotoxins (<10 kDa), Superdex Peptide HR 10/30 was chosen for its ability to separate peptides and small proteins below M_r 10.000. The fractions collected from the anion exchange chromatography that contained analgesic activity were pooled (fig. 2*a*), concentrated to 2 ml and then loaded on the Superdex Peptide HR 10/30 column (1.6 × 60 cm). The elution buffer was 20 mM phosphate buffered saline, pH 7.0, with 150 mM NaCl, and elution was carried out at a flow rate of 1.0 ml/min.

RP-HPLC. The fraction corresponding to the appropriate peak that was eluted from the gel filtration column was loaded onto a column of μRPC C2/C18 ST 4.6 × 100 mm. Gradient elution was carried out with solvent A (0.065% TFA, 5% acetonitrile in water) and solvent B (0.05% TFA, 70% acetonitrile in water) from 100% A to 100% B over 40 min at a flow rate of 0.7 ml/min.

SDS-PAGE and isoelectric focusing. Polyacrylamide gel electrophoresis was carried out using a 15% separation gel with a 5% stacking gel in a Tris-glycine buffer system. Sample volumes of 20 μl were loaded into each well. Constant currents of 10 mA at the stacking gel and 20 mA at the separation gel were applied. The gel was stained with Coomassie Brilliant Blue (CBB) R-250.

Isoelectric focusing (IEF) was carried out on a 4% polyacrylamide gel with 2% carrier ampholytes (pH range 3.5−10.5). The running buffers were 5%

H_3PO_4 (anode) and 2% NaOH (cathode), and a constant voltage of 150 V was applied. The gel was stained with CBB R-250.

HPCE. HPCE was performed on a JIANGSHEN system (Dalian, China). Samples were introduced via siphon mode into an uncoated fused silica (65 cm × 75 μm, inner diameter) capillary and run in 50 mM borax buffer, pH 7.5; experiments were performed under 15 kV from positive to negative polarity at 20°C for 40 min. Migration of the sample was monitored by UV spectrophotometry at λ = 280 nm.

Mouse writhing test. The mouse writhing test was performed as described previously [10]. Mice of 18−22 g were injected intraperitoneally with 0.2 ml of 0.9% (v/v) acetic acid per 20 g of body weight to induce extensive and long-lasting pain in their internal organs. The resultant twisting response of the mouse, reflecting pain intensity, was quantified. To perform the bioassay, 0.2 ml of analgesic fraction solutions were injected intravenously at the tail; 0.9% (w/v) NaCl was used as the negative control, and the pain killer morphine was used as the positive control. Twenty minutes later, 0.2 ml of 0.9% (v/v) acetic acid solution was injected intraperitoneally. Five minutes later, the number of twisting actions was counted for a 10-min period. Each group contained 10 mice, and the results were analyzed using a *t*-value test. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

Amino acid sequencing and cDNA cloning. The N-terminal 17 amino acids of the analgesic peptide were determined by automated Edman degradation with an Applied Biosystems Procise Sequencer. The phenylthiohydantoin (PTH) derivatives of amino acids were analyzed with an online PTH analyzer using a PTH-C18 column (ABI).
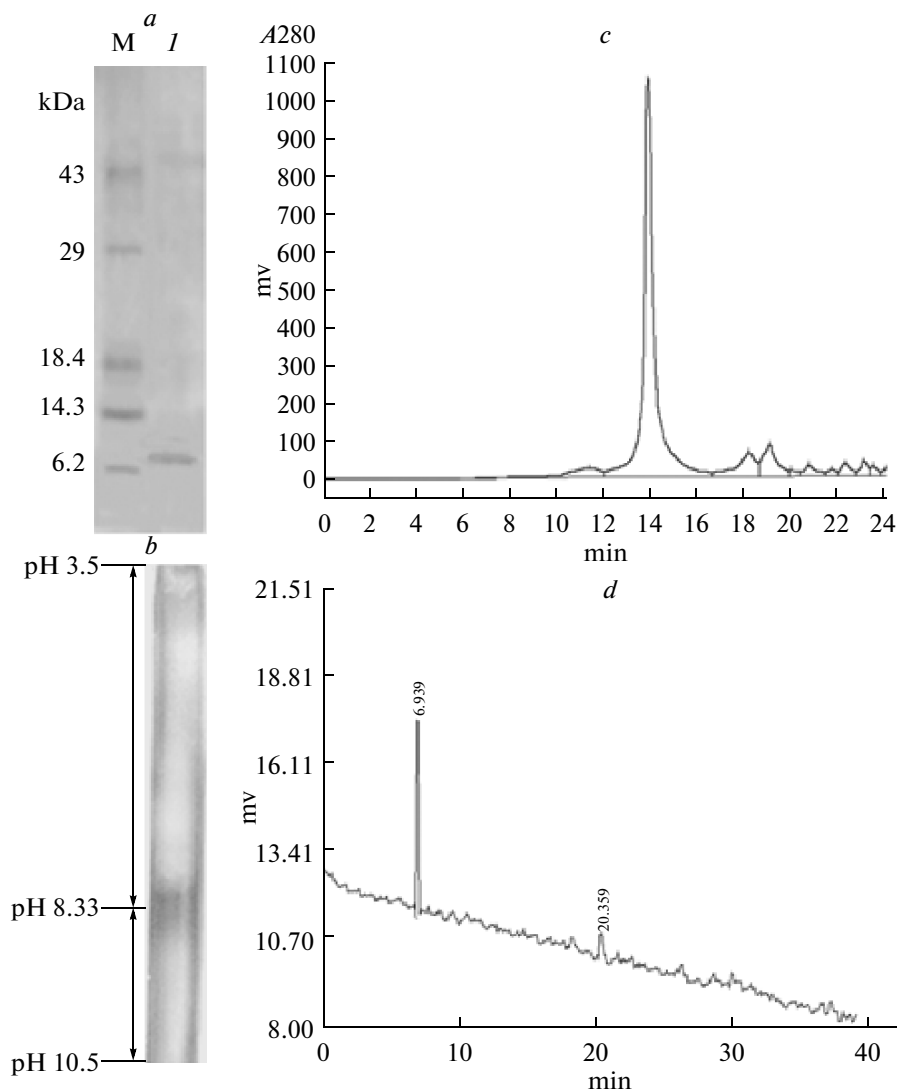
Fig. 3. Characterization of the analgesic peptide, BmK AGP-SYPU2. *a* − Coomassie Blue R250-stained SDS-PAGE gel (15%) of the purified analgesic peptide. Lane M: low molecular weight marker; Lane *1*: BmK AGP-SYPU2. *b* − Isoelectric focusing analysis of the purified analgesic peptide on a 4% polyacrylamide gel with 2% carrier ampholytes (pH range 3.5−10.5). *c* − RP-HPLC of the purified analgesic peptide on a μRPC C2/C18 ST column (4.6 × 100 mm). The sample was applied in 0.1% trifluoroacetic acid in water, and the column was eluted with a gradient of acetonitrile over 40 min. *d* − HPCE of the purified analgesic peptide.

The cDNA encoding the mature analgesic peptide was cloned by 3'-rapid amplification of cDNA ends (RACE). Total RNA (0.5 μg) was converted to cDNA using RevertAidTM M-MuLV reverse transcriptase and an oligodT$_{(17)}$ at 42°C for 60 min [11]. The partial gene encoding the analgesic peptide from the 3' end was then amplified by a pair of PCR primers: a gene-specific primer for the analgesic peptide, corresponding to the seven N-terminal residues (Val-Lys-Asp-Gly-Tyr-Ile-Ala), and oligodT$_{(17)}$. The PCR product was cloned into the pGEM-T Easy T Overhang Vector System, and positive clones were analyzed by nucleic acid sequencing [12].

RESULTS AND DISCUSSION

In recent decades, several hundreds of different scorpion polypeptides have been identified through biochemical purification or presumed from gene cloning methods [13]. Generally, most of the purification procedures have been carried out using RP-HPLC; consequently, upon elution with organic solvents, peptides may be partially or entirely denatured. To address the issue of denaturation of the scorpion peptides during the purification process, we have utilized biochemically temperate macromolecular separation and purification technologies including IEC, HIC and GF to

Analgesic activity at each purification step

Sample	Dose, mg/kg	Number of writhes, mean ± SD	Inhibition efficiency, %
Normal Saline	–	38.7 ± 3.9	–
Column (1a)-peak E	0.5	10.3 ± 4.0	73.5
Column (1b)-peak IV	1.0	5.1 ± 1.9	86.9
Column (1c)-peak V	0.1	15.1 ± 2.4	61.1
Column (2a)-peak II	0.1	14.0 ± 2.6	63.9
BmK AGP-SYPU2	0.1	13.8 ± 3.5	64.3
Morphine	1.0	13.5 ± 3.0	65.2

The inhibition efficiency is the ratio $(T_0 - T)/T_0$, in which T_0 is the mean number of writhes of the negative control group and T is the mean number of writhes of the experiment groups injected with analgesic fractions or the pain killer morphine ($n = 10$, $p < 0.01$ vs. Normal Saline).

purify the scorpion peptides from complicated venoms, which fully retain their proper bioactivity.

Every separation and purification step was monitored and screened out by an analgesic activity bioassay, and the most potent of analgesic fractions was chosen for the next separation (data not shown). The analgesic fraction which has been selected for the next purification step was marked by a corresponding bar in fig. 1 and fig. 2. BmK AGP-SYPU2 was obtained by a five-step separation and purification protocol that included cation exchange chromatography (fig. 1*a*, *b*), HIC (fig. 1*c*), anion exchange chromatography (fig. 2*a*) and GF (fig. 2*b*). The purity of the analgesic peptide was determined by SDS-PAGE (fig. 3*a*), RP-HPLC (fig. 3*c*) and HPCE (fig. 3*d*). From these results we can conclude that BmK AGP-SYPU2 was purified to homogeneity. The isoelectric point of the analgesic peptide was determined to be 8.33 by IEF (fig. 3*b*), and the apparent molecular weight was determined to be about 7.4 kDa.

As summarized in the table, the mouse writhing test was used to determine the analgesic activity of BmK AGP-SYPU2 and the purified fractions *in vivo*. The fraction with the highest analgesic activity was chosen for the next step in the chromatographic process; however, we found that the dose required to produce equivalent analgesic effects did not decrease with the increasing purity of the sample (table). This phenomenon exists in most scorpion toxins, such as BmK AGP-SYPU1 [9], BmK AS [14]; it is generally acknowledged that scorpion venom contains a wide variety of compounds, such as mucopolysaccharides, hyaluronidase, phospholipase, low relative molecular mass molecules like serotonin and histamine, protease inhibitors, histamine releasers and polypeptides [15], and it is possible that the venoms contain both direct-acting and synergistic components. A common basis is their occurrence in the venom together with hyaluronidase, which acts in a general manner on a wide range of different cell membranes [16]. When the synergistically acting components are separated, the peptides exhibit reduced activity.

In this study, the classic testing model was used to detect the analgesic effect of purified BmK AGP-SYPU2 on whole animals. In the mouse writhing test, mice were injected intraperitoneally with acetic acid to induce pain in their internal organs. In our preliminary pharmacological studies, BmK AGP-SYPU2 inhibited 64.3% of the twisting action induced in the mice by acetic acid at a dose of 0.1 mg/kg body weight (table). Thus, BmK AGP-SYPU2 exhibits a strong analgesic effect on visceral pain. Previously, our group has reported the purification of BmK AGP-SYPU1 from the same venom, which inhibited 70% of the twisting action at a dose of 0.1 mg/kg body weight (data not shown).

The first 17 residues of the N-terminus of the purified analgesic peptide, Val-Lys-Asp-Gly-Tyr-Ile-Ala-Asp-Asp-Arg-Asn-Cys-Pro-Tyr-Phe-Cys-Gly, were determined by Edman degradation. Based on the first seven residues, we cloned the corresponding 198 bp cDNA (fig. 4*a*) that encoded a mature peptide of 66 residues (fig. 4*b*). In the NCBI Sequence Blast, this analgesic peptide was found to be identical to BmK α2

a   *b*


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1    GTA AAG GAT GGT TAT ATT GCT GAC GAT AGA AAC TGC CCA TAC TTT
1    V   K   D   G   Y   I   A   D   D   R   N   C   P   Y   F

46   TGT GGT AGA AAT GCA TAT TGC GAT GGA GAA TGT AAG AAG AAC CGT
16   C   G   R   N   A   Y   C   D   G   E   C   K   K   N   R

91   GCT GAG AGT GGC TAT TGC CAA TGG GCA AGT AAA TAC GGA AAC GCC
31   A   E   S   G   Y   C   Q   W   A   S   K   Y   G   N   A

136  TGC TGG TGC TAT AAG TTG CCC GAT GAT GCA CGT ATT ATG AAA CCA
46   C   W   C   Y   K   L   P   D   D   A   R   I   M   K   P

181  GGA AGA TGC AAT GGC GGT
61   G   R   C   N   G   G
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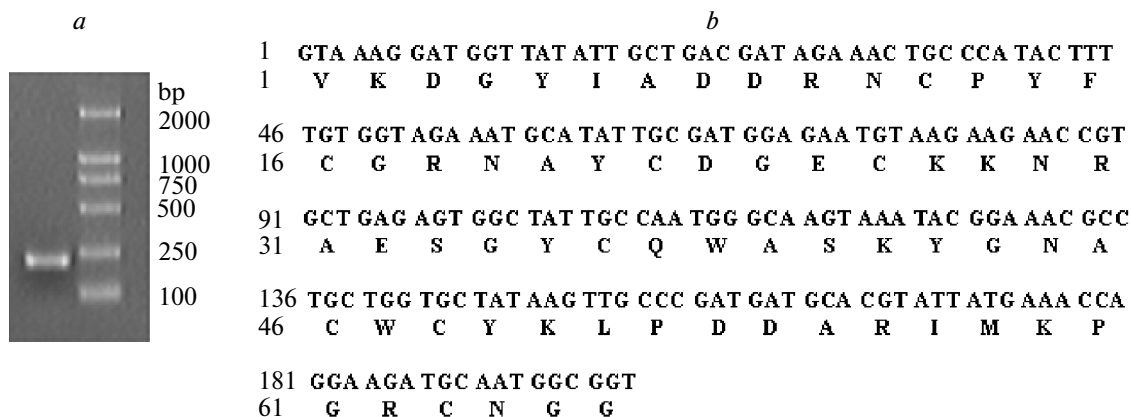
Fig. 4. Analysis of the amplified cDNA encoding BmK AGP-SYPU2 on a 1.5% agarose gel (*a*) and the nucleotide and deduced amino acid sequences of BmK AGP-SYPU2 (*b*).

and BmK αTX11, which were identified previously [17, 18]. Despite this whether BmK α2 and BmK αTX11 have analgesic activity has not been investigated earlier.

SNX-III, the only peptide of a novel calcium channel blocker, has been used clinically as pain killer [19]. Scorpions have been used in traditional Chinese medicine for thousands of years, and the safety and efficacy of these natural products have been confirmed by many studies [20, 21]. From the results of this study, it can be concluded that BmK AGP-SYPU2, found in Chinese scorpion venom, may be a new analgesic peptide that may serve as a useful candidate in search for a potential treatment for analgesia.

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