Electronic supplementary materials *Mendeleev Commun.*, 2022, **32**, 745–746

Optimized method for the recombinant production of a sea anemone's peptide

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1. The producent strain *E. coli Bl21(DE3)/pSMT3_HCRG21* construction

The fusion protein SMT3-HCRG21 sequence (Figure S1) was synthesized as a nucleotide sequence with the codon optimization for prokaryotic expression system.

MVKYHHHHHHGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSS
EIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHR
EQIGGRGICSEPKVVGPCTAYFRRFYFDSETGKCTPFIYGGCEGNGNNFETLRACRAICR
A*stop*

Figure S1 The sequence of the fusion protein. Blue color shows N-terminal sequence of Smt3, red color shows HCRG21 sequence, the leading peptide with incorporated His-tag (underlined) is colorless.

The SMT3-HCRG21 nucleotide sequence was inserted into a hybrid vector based on pET23 and pET28 vectors, resulting in the pSMT3_HCRG21 plasmid, which contained the par101 toxin-antitoxin system, the kanamycin resistance gene, and necessary structural elements (Figure S2). The plasmid construction is available from the authors upon request.

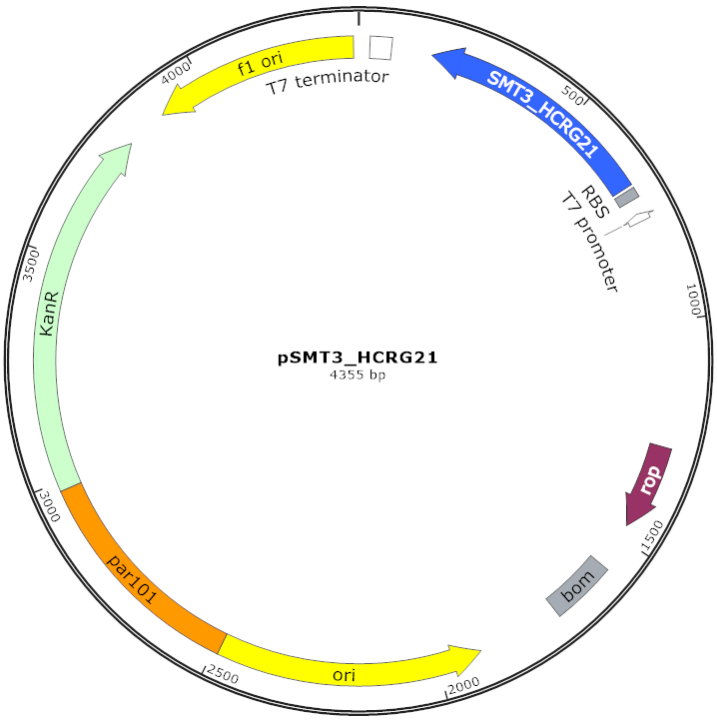

Figure S2 Plasmid pSMT3_HCRG21 map.

The producent strain *E. coli BL21 (DE3)/pSMT3_HCRG21* was obtained via the transformation of competent cells *E.coli BL21 (DE3)* with pSMT3_HCRG21 plasmid vector. In brief, 1.5 µL of the plasmid was added to 100 µL of chemically competent cells *E. coli BL21 (DE3)* and kept 30 min in an ice bath followed by 45 sec incubation at 42°C. The cells were kept 5 min in the ice bath again, resuspended in 300 µL of SOC media and cultivated for 1 hour on a shaker (225 rpm) at 37°C.

The test tubes content was sown on Petri dishes with LB-agar containing a selective antibiotic kanamycin (50 mg/L) and incubated for 14-16 hours at 37°C. The single colonies were harvested to obtain a stock culture by cultivation in 15 mL tubes using LB nutrient medium containing 50 mg/L of kanamycin and 0.5% glucose (190 rpm, 37°C, 15-16 hours). The resulting culture was transferred 2.5% by volume to a fresh tube with LB nutrient medium containing 50 mg/L of kanamycin and 0.5% glucose and cultured up to OD_{600} of 0.6-0.7 units (190 rpm, 37°C). Finally, glycerin was added up to 15% by volume and the resulting research bank was stored at -70°C.

2. Fermentation

Museum culture 0.1% was inoculated in LB medium containing 50 mg/L of kanamycin and 5 g/L of glucose. After 16 hours of incubation (180 rpm, 32°C), the absence of extragenic microflora in the culture was checked by microscopy. Cells were grown in a 30-liter fermenter (MBR, Switzerland) in 20 liters of LB medium at 37°C. The culture medium was monitored every hour for pH value, amount of oxygen, optical density at 600 nm, and glucose content. Upon reaching OD_{600} to 12, the air consumption was decreased from 25 to 15%, and 0.3 g of lactose was injected into the fermenter for the induction of biosynthesis. After that, fermentation was continued for 60 minutes at 37°C. In the end, wet biomass (1 kg) was collected after centrifugation of the culture medium at 10000 g for 20 minutes at 10°C.

The resulting biomass was resuspended in 50 mM Tris-HCl, 20 mM EDTA, pH 8.0 buffer using 10 mL of the buffer per 1 g of biomass, after that, cells were disrupted with APV Gaulin MC 18-3TPS homogenizer. The resulting cell lysate was centrifuged at 10000 g for 20 minutes at 10°C. The pellet (70 g) was resuspended in 50 mM Tris-HCl, pH 8.0 buffer, stirred for 20 minutes and finally precipitated at 10000 g for 20 minutes at 10°C.

3. HCRG21 purification

HCRG21 was synthesized in *E. coli* strain *Bl21(DE3)* where it accumulated inside cells as an insoluble cellular precipitate (the step-by-step purification protocol is given below). The content of the fusion protein of interest in the washed pellet was estimated as 90% according to the densitometric analysis of gels in the Gelanalyzer program. The fusion protein was extracted by the extraction buffer (50 mM Tris-HCl, pH 8.0 with 8 M urea), purified by metal-chelate chromatography on Ni-containing resin, and renatured at 4ºC overnight. Initially, immediately after renaturation, Ulp1 protease was added to the sample and the fusion protein was hydrolyzed at room temperature for 3 hours (Figure S3).

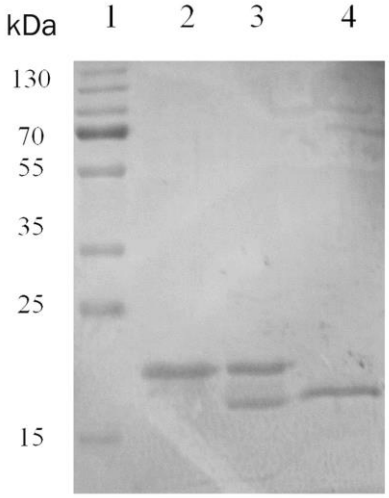

Figure S3 12.5% SDS PAGE under reducing conditions. Line 1 – molecular weight markers; line 2 – SMT3-HCRG21 after Ni-containing resin but before renaturation; line 3 – SMT3-HCRG21 after renaturation; line 4 – hydrolyzate of SMT3-HCRG21 by Ulp-1 (3h at room temperature).

Fusion protein SMT3-HCRG21 was completely cleaved by a specific protease for 3 hours (Figure S3 line 4), but the presence of additional bands (Figure S3 line 3) was observed in the sample at the end of renaturation, which could be the result of the fusion nonspecific proteolysis by endogenous cellular proteases. The formation of excess impurities during purification process is unacceptable, therefore, in addition to first purification by metal-chelate chromatography, an anion exchange chromatography stage on DEAE-Sepharose was added. The fusion purified on DEAE-Sepharose remained stable during renaturation and no additional bands on electrophoresis were observed.

HCRG21 peptide was purified from hydrolysate by repeated application of the mixture on a metal-chelate resin, which retained all reaction mixture components except for the target peptide. The sample had insufficient purity as shown by analytical reverse-phase HPLC. The peak of the interest (elution time about 8 minutes) by relative area did not exceed 26% (Figure S4).

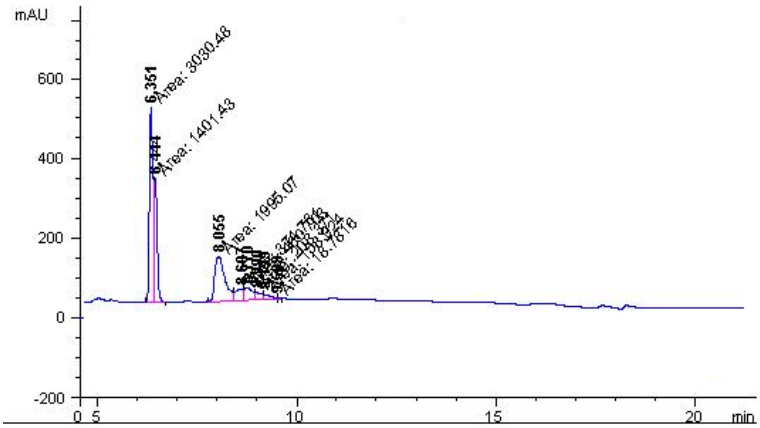

Figure S4 The chromatographic analysis of HCRG21 purity after the second affinity column purification stage on an analytical column VDSpher PUR 100 C18-E (VDS, Germany) 250x4.6 mm. Elution at 1 mL/min flow was done in a linear gradient of acetonitrile concentration from 5 to 90% in the presence of 0.1% TFA. Areas were calculated for absorbance at 214 nm.

For final purification, the target peptide was subjected to semi-preparative RP-HPLC on ODS-A media from YMC, which allowed us to obtain the target product with the purity of 96.7% (Figure S5) and the total yield of 10.2 mg per 1 liter of culture. According to mass spectrometry data, the average molecular weight of the product, 6228.8 Da, (MALDI MS in a linear mode) corresponded well to the calculated weight, 6228.09 Da, for the peptide with three disulfide bonds (Figure S6).

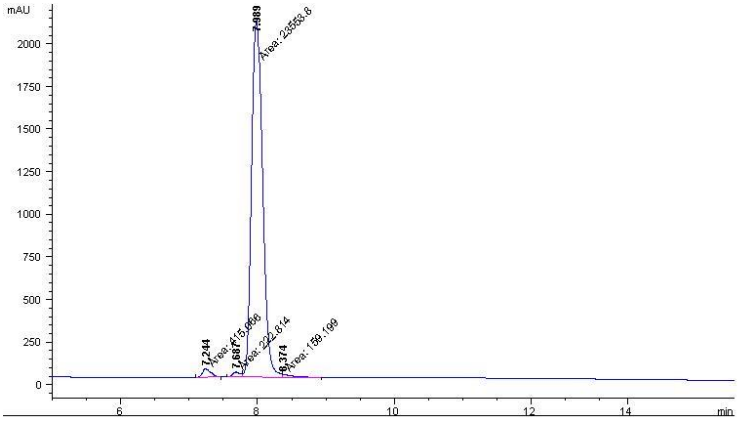

Figure S5 The chromatographic analysis of HCRG21 purity after the semi-preparative RP-HPLC on an analytical column VDSpher PUR 100 C18-E (VDS, Germany) 250x4.6 mm. Elution at 1 mL/min flow was done in a linear gradient from 5 to 90% acetonitrile concentration in the presence of 0.1% TFA. Areas were calculated for the absorbance at 214 nm.

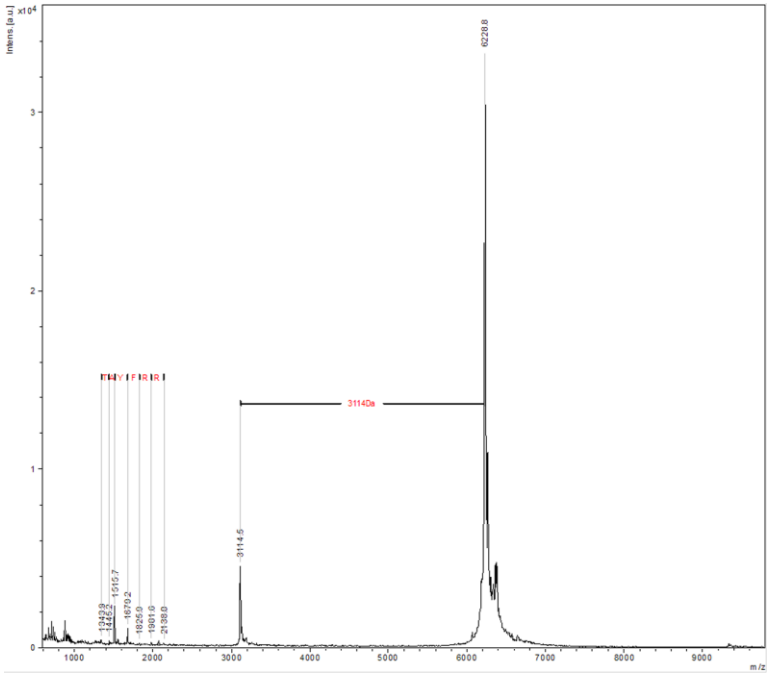

Figure S6 MALDI spectrum of HCRG21.

<u>Detailed protocol for the peptide purification was drawn up.</u>

Washed insoluble cellular pellet was dissolved in 50 mM Tris-HCl, pH 8.0 buffer with 8 M urea using 10 mL of buffer per 1 g of pellet. Extraction was carried out for 30 minutes on a magnetic stirrer, further, supernatant was collected after the centrifugation at 10000 g for 20 minutes at 10°C.

For metal-chelate chromatography, XK 50 column with 300 mL of Ni IMAC SepFast media (BioToolomics, England) and Akta Pure chromatographic system (Cytiva) were used. The media was pre-activated by two column volumes of 0.15 M $NiSO_4$ solution. The flow rate was set to 17 mL/min. Buffer A contained 50 mM Tris-HCl, pH 8.0, 8 M urea. Buffer B was buffer A supplemented with 500 mM imidazole. After the application of supernatant, the column was washed with buffer A until an UV absorption at the wavelength of 280 nm and a conductivity reached a plateau. The fusion protein elution was carried out in a linear gradient 0-100% of buffer B for 120 minutes.

Next, SMT3-HCRG21 was purified using anion exchange chromatography on XK 50 column with 200 mL of DEAE-Sepharose media (GE Healthcare) and Akta Pure chromatographic system. The flow rate was 17 mL/min. Buffer A contained 50 mM Tris-HCl, pH 8.0, 2 M urea. Buffer B was buffer A supplemented with 1 M NaCl. After the application of the sample, the column was washed with buffer A until an absorption at the wavelength of 280 nm and a conductivity reached a plateau. The elution was carried out in a linear gradient 0-100% of buffer B for 80 minutes.

The eluate obtained after DEAE-Sepharose was renatureted by 2-fold dilution with a renaturation buffer (50 mM Tris-HCl, pH 8.5) at 4°C overnight, with shaking at 100 rpm. After that, a suspension of lyophilized Ulp-1 protease was added to the SMT3-HCRG21 renaturant (1 mg of enzyme per 100 mg of fusion protein) together with 1 M dithiothreitol solution up to 1 mM final concentration. The digestion was carried out with stirring for at least 3 hours at room temperature, after that, the reaction mixture was applied on a column with 5 mL of Ni IMAC SepFast (media and conditions are similar to the described above) and a non-bound fraction was collected.

For the final purification, 20 mL of ODS-A media (YMC, Japan) packed in a 1 cm diameter column and Akta Pure chromatographic system were used. Buffer A contained 0.1% acetic acid, 2% ethanol, and Buffer B contained 0.1% acetic acid, 75% ethanol. The column was equilibrated with buffer A, and the non-bound on Ni-column fraction was applied, after that, the column was washed with buffer A until an UV absorption at the wavelength of 280 nm and a conductivity reached a plateau. The elution was carried out in a linear gradient 0-100% of buffer B for 50 minutes at a flow rate 2 mL/min. The collected fractions were analyzed using analytical HPLC for the impurities measure (%). The molecular weight of the pure peptide was determined by MALDI mass spectrometry.

4. Confirmation of HCRG21 activity

CHO (Chinese hamster ovary) cells were obtained from Evrogen company (http://evrogen.ru). The CHO cell line stably expressing rat TRPV1 was generated using T-Rex System (Invitrogen) according to the manufacturer's instructions. Briefly, the vector pcDNA4/TO with cDNA encoding rat TRPV1 was made, and transfected into CHO cells carrying the regulatory vector pcDNA6/TR (encoding the tetracycline repressor). The CHO cells were grown at 37°C and 5% CO_2 in DMEM/F12 (1:1) medium containing 10% fetal calf serum. After 2 weeks of selection by blasticidin (5 μg/mL) and zeocin (250 μg/mL), single colonies were screened using the capsaicin-induced [Ca^{2+}] uptake assay. CHO cells stably expressing rat TRPV1 were sown into black-walled, clear-bottomed 96-well plate at the density of 40,000 cells per well (complete media without antibiotics and containing 1 μg/mL tetracycline to induce channel expression) and were cultured overnight at 37°C and 5% CO_2. The cells were then loaded with 20 μL per well of voltage sensitive dye from FLIPR Membrane Potential Assay Kits (Molecular Devices, San Jose, USA) (Cat #R8126) in accordance with the manufacturer's protocol and were incubated for 30-40 min in CO_2 incubator (dark, 37ºC). The water (control) or HCRG21 peptide was added to the well 5 minute before the measurement.

Image of cells in a well was recorded at 40x objective lens before and after the addition of TRPV1 agonist capsaicin to the final concentration 1 μM. Camera was mounted on Leica DM IL LED Fluo microscope equipped with G/R filter cube: EX: 225/-450 | DC: 495 595 | EM: 250/-500. Relative fluorescence was calculated for whole image after background subtraction as the fluorescence of the activated cells divided to the fluorescence of the same cells before the activation.