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Mendeleev Commun., 2022, **32**, 745–746

Mendeleev Communications

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

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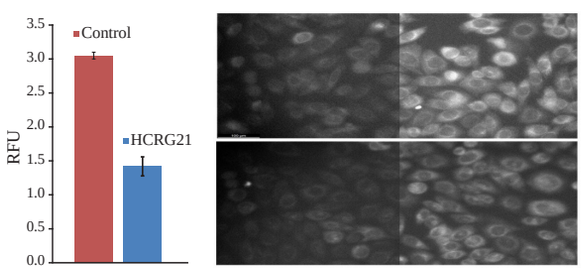
DOI: 10.1016/j.mencom.2022.11.012

The production of the analgesic peptide HCRG21 for medical use is restricted by a number of limitations in the technology. The optimal biotechnological method scalable to industrial has been developed based on the production of a fusion protein containing a special leader, His-tag, and Smt3 sequence upstream in the peptide sequence. The resulting peptide shared its inhibitory activity to TRPV1 ion channel identical to the published early.



Keywords: analgesic peptide, biotechnology, SUMO fusion, production in prokaryotes, fluorescence imaging, TRPV1 ion channel.

The peptide HCRG21 (UniProt ID: P0DL86), whose amino acid sequence was established by analyzing transcripts encoding Kunitz-type peptides of the sea anemone *Heteractis magnifica* (early published as *Heteractis crispa*),[1] demonstrated attractive biological effects in different *in vivo* models.[2,3] Acting as a potent inhibitor of TRPV1 ion channel,[1] this molecule has prospects for both fundamental research and medicine when the activity of the TRPV1 ion channel should be temporarily reduced. So far, a low molecular weight antagonist capsazepine is used in such cases, but it has low affinity and selectivity.[4,5] The second important advantage of the peptide over other known TRPV1 inhibitors is the long duration of the effect, which was shown in hot plate experiments on mice.[2] HCRG21 exhibited analgesic effect for at least 13 h at the dose of 0.1 mg kg^{-1} upon intramuscular administration. These peculiarities allow us to consider HCRG21 for further drug development if the effectiveness, biosafety and bioavailability of the peptide will first be confirmed in preclinical trials. Therefore, the development of the peptide's synthesis method characterized by high yield, safety and scalability to semi-industrial scale is relevant.

For therapeutic peptide synthesis, the preservation of the original amino acid sequence from modifications is especially important; therefore, the development of the strategy is not an easy task. Typically, such peptides are synthesized as fused proteins containing, in addition to the target peptide, different auxiliary sequences, such as affinity tags, carrier proteins to increase target peptide's productivity or solubility (thioredoxin, maltose-binding domain, glutathione *S*-transferase, *etc*.), as well as other supplementary peptides.[6,7] However, the presence of any assistant sequences or their parts in the sequence of a target peptide drug is unacceptable. To solve this problem, fusion proteins are usually treated with specific proteases which recognize conservative amino acid sequences located just before the target peptide. Unfortunately, only a limited number of mammalian enzymes such as blood clotting factor Xa, enterokinase, α-thrombin, SUMO-protease or viral enzymes such as human rhinovirus protease 3C and tobacco engraving virus protease (TEV) are used in biotechnology so far.[8–13]

First amino acid residue, arginine, in HCRG21 sequence makes it impossible to use both TEV protease and factor Xa. Enterokinase, that is tolerant to any amino acids at the P1' position of the cleavage site, often causes a nonspecific hydrolysis of R/K-rich substrates.[10] We decided to use a yeast Ubl-specific protease, Ulp1, which cleaves proteins from Smt3 and SUMO-1.[14] Since Smt3 leader sequence increases stability, solubility and yield of the fusion protein, this sequence was chosen as a partner for Ulp1 protease.[12]

For HCRG21 production in prokaryotic expression system, the SMT3–HCRG21 fusion protein with several features (sequence shown in Online Supplementary Materials) was constructed. The fusion protein included a leader sequence of 21 amino acid residues for biosynthesis boost, which also contained His-tag for metal-chelate chromatography, followed by SUMO protein Smt3 partial sequence of 98 residues up to paired glycines, and C-terminal part identical to the HCRG21 peptide.

To reduce the antibiotic consumption at the fermentation stage and overall cost of production, a toxin–antitoxin pair was included in the pSMT3_HCRG21 plasmid. This system based on the natural variant of hok/sok from *Escherichia coli* ensures the culture purity due to the lethal action of the peptide toxin (hok) which is encoded in the plasmid.[15] Simultaneously, the antisense RNA (sok) that inhibits the toxin production is also transcribed from the plasmid, which guarantees the survival of producent cells containing pSMT3_HCRG21. *E. coli* Bl21(DE3) was

selected as the producent strain, in which biomass production was carried out at 30-liter fermenter scale.

The fusion protein production in *E. coli* Bl21(DE3) led to the formation of an insoluble cell precipitate, where the target product was stored [Figure 1(*a*)]. This positive result allowed us to increase the overall yield of a product and to protect the fusion protein from the degradation by cellular endogenous proteases.

The fusion protein (about 90% purity) was obtained by the metal-chelate chromatography on Ni IMAC SepFast resin after dissolving a thoroughly washed insoluble cell precipitate in 8 M urea [Figure 1(*b*)]. The target peptide is the disulfide-stabilized protein with a Kunitz-type fold, wherefore, the renaturation was necessary for the correct spatial structure formation. The renaturation was performed for the whole fusion-protein overnight in the cold, after that, the hydrolysis by Ulp1 protease (in-house production) was done. The substrate was hydrolyzed completely in 3 h, however, nonspecific proteolysis by host cellular proteases was observed (see Online Supplementary Materials). The stability of the renatured fusion in solution was restored after the introduction of an additional purification stage on the anion-exchange media DEAE-Sepharose after the separation on Ni IMAC SepFast column.

Further HCRG21 purification was based on the reuse of the Ni IMAC SepFast column, that retained Smt3-leader peptide, non-cleaved hybrid protein, and Ulp1-protease that contained the His-tag as well, but did not bind HCRG21. The final purification stage was carried out by a semi-preparative RP-HPLC on YMC ODS-A media (separation details given in Online Supplementary Materials). As a result, HCRG21 was separated from impurities and the target product with about 97% of purity and the yield of 10 mg per liter of culture was obtained. In literature, another approach using DnaB mini-intein to obtain homologous peptides in a prokaryotic expression system was described. In the work of Esipov and co-authors,[16] APHC3 toxin from *H. crispa* was obtained using two chromatographic purification stages with the yield of 11.5 mg dm^{-3}, which is comparable to the methodology proposed in this work.

The activity of HCRG21 peptide was evaluated by fluorescent imaging on the cell line stable expressing TRPV1 ion channels (Figure 2). At the concentration of 5 μM, HCRG21 inhibited 50% relative fluorescence of cells activated by 1 μM capsaicin. This activity was similar to the activity measured earlier for this peptide,[1] where 6.9 μM HCRG21 inhibited capsaicin-induced current in *Xenopus* oocytes to 50%.

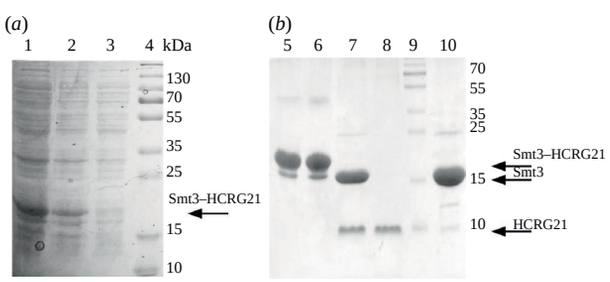


Figure 1 SDS-PAGE analysis of HCRG21 production. (*a*) Cell lysate after *E. coli* Bl21(DE3)/pSMT3_HCRG21 biomass degradation in 15% gel. (*b*) Target peptide purification stages in 12.5% gel. Line 1 – total cell lysate; line 2 – insoluble cell precipitate; line 3 – soluble cell lysate; lines 4 and 9 – molecular weight markers; line 5 – a fraction from the first metal-chelate chromatography; line 6 – SMT3–HCRG21 after renaturation; line 7 – the fusion protein proteolysis by Ulp-1 (3 h); line 8 – a non-bound fraction at the second metal-chelate chromatography; line 10 – a bound fraction at the second metal-chelate chromatography.

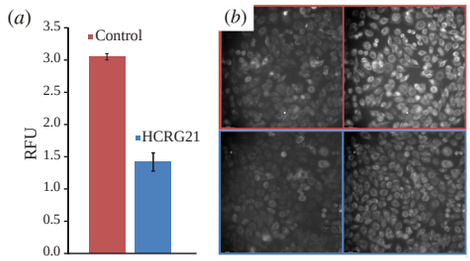


Figure 2 Fluorescent analysis of HCRG21 activity. (*a*) Relative fluorescence calculation as F/F0-1 for 3 independent pairs. (*b*) Images used for the analysis, F0 (left) and F (right). The peptide in 5 μM concentration was preincubated for 5 min with CHO-TRPV1 cells (panel (*b*) bottom), the same volume of water was added to the control cells (panel (*b*) top). The TRPV1 ion channels were activated by capsaicin addition to 1 μM final concentration into the well.

As a result of the work, we have developed a technology for obtaining the biologically active sea anemone peptide in the prokaryotic expression system, which ensures its functionality and purity strategically forethought for the use in pharmaceutical applications. The technique can be reproduced, is cost-effective and scalable up to 10–50 g of API per a production cycle.

This work was supported by the Far Eastern Federal University (Program ‘PRIORITY 2030’: Life Science).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2022.11.012.

References

1 M. Monastyrnaya, S. Peigneur, E. Zelepuga, O. Sintsova, I. Gladkikh, E. Leychenko, M. Isaeva, J. Tytgat and E. Kozlovskaya, *Mar. Drugs*, 2016, **14**, 229.
2 O. V. Sintsova, V. A. Palikov, Y. A. Palikova, A. A. Klimovich, I. N. Gladkikh, Y. A. Andreev, M. M. Monastyrnaya, E. P. Kozlovskaya, I. A. Dyachenko, S. A. Kozlov and E. V. Leychenko, *Dokl. Biochem. Biophys.*, 2020, **493**, 215 (*Dokl. Ross. Akad. Nauk, Nauki o Zhizni*, 2020, **493**, 423).
3 O. Sintsova, I. Gladkikh, A. Klimovich, Y. Palikova, V. Palikov, O. Styshova, M. Monastyrnaya, I. Dyachenko, S. Kozlov and E. Leychenko, *Biomedicines*, 2021, **9**, 716.
4 R. J. Docherty, J. C. Yeats and A. S. Piper, *Br. J. Pharmacol.*, 1997, **121**, 1461.
5 L. Liu and S. A. Simon, *Neurosci. Lett.*, 1997, **228**, 29.
6 S. T. Loughran and D. Walls, *Methods Mol. Biol.*, 2017, **1485**, 131.
7 Yu. A. Logashina, Yu. V. Korolkova, E. E. Maleeva, D. I. Osmakov, S. A. Kozlov and Ya. A. Andreev, *Mendeleev Commun.*, 2020, **30**, 214.
8 F. Cesaratto, O. R. Burrone and G. Petris, *J. Biotechnol.*, 2016, **231**, 239.
9 R. J. Jenny, K. G. Mann and R. L. Lundblad, *Protein Expression Purif.*, 2003, **31**, 1.
10 M. E. Gasparian, M. L. Bychkov, D. A. Dolgikh and M. P. Kirpichnikov, *Protein Expression Purif.*, 2011, **79**, 191.
11 M. G. Cordingley, P. L. Callahan, V. V. Sardana, V. M. Garsky and R. J. Colonno, *J. Biol. Chem.*, 1990, **265**, 9062.
12 F. Guerrero, A. Ciragan and H. Iwaï, *Protein Expression Purif.*, 2015, **116**, 42.
13 W. Lu, X. Cai, Z. Gu, Y. Huang, B. Xia and P. Cao, *Mol. Biotechnol.*, 2013, **53**, 41.
14 S. J. Li and M. Hochstrasser, *Nature*, 1999, **398**, 246.
15 S. Masachis and F. Darfeuille, *Microbiol. Spectrum*, 2018, **6**, DOI: 10.1128/microbiolspec.RWR-0030-2018.
16 R. S. Esipov, D. A. Makarov, V. N. Stepanenko, M. A. Kostromina, T. I. Muravyova, Y. A. Andreev, I. A. Dyachenko, S. A. Kozlov and E. V. Grishin, *Protein Expression Purif.*, 2018, **145**, 71.

Received: 5th October 2022; Com. 22/6982