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Recombinant approach for the production of HIV fusion inhibitor Enfuvirtide using *Escherichia coli*

Ravikanth Reddy Kosana [a,b], Chitra Bajji [a], Radha Madhavi Kanumuri [a], Kalpana Panati [c], Lakshmi Narasu Mangamoori [b], Muralikrishna Reddy Tummuru [a], Venkata Ramireddy Narala [d,*]

[a] *Virchow Research Center, Hyderabad 500 043, AP, India*
[b] *Center for Biotechnology, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad 500 085, AP, India*
[c] *Department of Biotechnology, Government Degree College for Men, Kadapa, AP, India*
[d] *Department of Zoology, Yogi Vemana University, Kadapa 516 003, AP, India*

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ABSTRACT

The use of antiretroviral drugs is gaining importance in the recent past for the treatment of human immunodeficiency virus infection. Enfuvirtide (T20) is one of the fusion inhibitors, inhibits the fusion between the virus and healthy target CD4 cells. The treatment with T20 involves very high therapeutic dose. In addition to its high dose, production of T20 by synthetic methods is expensive and cumbersome. We report an alternative recombinant approach for the production of the T20 peptide through a novel short fusion-tag expression system. This expression system consists of the hydrophobic region of growth hormone (GH) as the fusion tag, a factor Xa cleavage site upstream to the T20. The fusion protein was expressed in *Escherichia coli* as inclusion bodies. We also report here, a simple and an efficient downstream strategy for the purification of recombinant T20 peptide (rT20). Our study is the first to demonstrate a novel approach using GH fusion tag, ensured the peptide stability, for the production of rT20 which yields more than 250 mg/L with 98% purity. The biological activity of the rT20 is comparable to its synthetic counterpart. Thus, this novel approach could be an alternate method of choice for production of therapeutically important small peptides.

Introduction

AIDS is a disease of human immune system and considered as a pandemic. Globally, 34 million people were infected with HIV and more than 25 million people died due to HIV/AIDS (www.aids.gov). So far 31 anti-HIV drugs and combination therapies have been approved for clinical use (www.fda.gov). Most of them are either reverse transcriptase or protease inhibitors. Use of these drugs in combination therapies, commonly known as highly active antiretroviral therapy [1,2], significantly reduced the morbidity and mortality of HIV patients. However, the success of the current therapy is limited due to the rapid emergence of drug resistant mutant viruses [3]. This has prompted the search for new drug candidates, which are safe and highly effective with low risk of cross-resistance. Enfuvirtide (T20), a synthetic peptide with 36-amino acids with molecular weight of 4.5 kDa, is one of such new drugs that targets viral entry. T20 inhibits the fusion between virus and target CD4 cells [4,5] and is the first antiretroviral drug of its class, approved by USFDA for HIV infected patients who have failed to respond to the current antiretroviral drugs [5,6].

The HIV-1 viral envelope glycoprotein (gp160) consists of two non-covalently associated subunits, a surface glycoprotein (gp120) that is responsible for viral binding to the receptors and a transmembrane glycoprotein (gp41) that mediates virus fusion and entry [7]. During HIV infection, gp120 binds to CD4 and a chemokine receptor CCR5 on the target cell to trigger gp41 structural rearrangement. This results in the formation of a stable gp41 six-helix bundle (6HB) core structure, consisting of 3 N-terminal helices, which are termed as Heptad Repeat region-1 (HR-1)[1] and 3 C-terminal helices, which are termed as Heptad Repeat region-2 (HR-2). This structural change brings both the viral and target cell membranes into proximity resulting in fusion between the virus and the target cell membranes [8,9]. T20 is a 36 amino acid peptide that is similar to the sequence of the HR-2 region (638–673) of the gp41 glycoprotein. It inhibits virus-cell fusion by binding to the HR-1 region of gp41 glycoprotein preventing the formation 6-helix bundle and thus arrests the viral entry [10].

* Corresponding author. Tel.: +91 8562 225498; fax: +91 8562 225419.
E-mail address: nvramireddy@gmail.com (V.R. Narala).

[1] *Abbrevations used:* HR-1, Heptad Repeat region-1; HR-2, Heptad Repeat region-2; GH, growth hormone; rT20, recombinant T20 peptide; FXa, factor Xa; IB, inclusion bodies; LB, Luria–Bertani medium; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; IC_{50}, inhibitory concentration value; RPC, reversed phase chromatography; KSI, ketosteroid isomerase.

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At present, the only approach available to produce T20 peptide is by the chemical synthesis method [11]. This synthetic method is very cumbersome and expensive. In this report, we have shown that the recombinant approach for the production of T20 peptide which is economically viable and alternative to synthetic method. In this approach we have also reduced the ratio of fusion tag to the peptide of interest with a hope to increase the stability and the yield of the expressed protein. We have used N-terminal region (1–62 aa) of synthetic human growth hormone (GH) [12] as a fusion partner for the over expression of T20 peptide. In addition, factor Xa (FXa) protease cleavage site was introduced for a facile cleavage of rT20 peptide from the fusion tag. This is the first report of such reduced size fusion tag. The fusion protein (GH-T20) was expressed in the form of inclusion bodies (IB). From IBs, the fusion protein was cleaved with FXa to release the rT20 peptide. This cleaved rT20 peptide was purified by reversed phase chromatography in high yields (~250 mg/L) with purity greater than 98%.

Materials and methods

Materials

Plasmid (pET21a) and *E. coli* host cells (Rosetta) were obtained from Novagen (Darmstadt, Germany). Source-30™ reversed phase chromatography matrix from GE Healthcare (Germany) was used for purification of the peptide. Thermo cycler from Techne Ltd. (Cambridge, U.K.) was used for PCR amplification. The T20 gene and PCR primers were synthesized by IDT Technologies (Coralville, Iowa, USA).

High pressure homogenizer (Model: NS100L Panada) from GEA Niro Soavi (Italy) was used for cell lysis. AKTA pilot chromatography system (GE Healthcare, Germany) was used for the pilot-scale purification. The Enfuvirtide standard 'Fuzeon' was purchased from Roche Pharmaceuticals (New Jersey). FXa was isolated in-house from bovine plasma and was activated by a protease from Russell's viper venom.

Methods

T20 gene construction

Based on the known sequence of HR2 domain of gp41 glycoprotein (GenBank: AAA76666.1) (from 638 to 673 amino acid regions) and the codon preference of the *E. coli*, the following complete monomer (108 bases) template was designed. This template was synthesized by IDT Technologies (Coralville, Iowa, USA).

5′-TACACCAGCCTGATCCACAGCCTGATCGAAGAAAGCCAGAACCAGCAGGAAAAAAACGAACAGGAACTGCTGGAACTGGACAAATGGGCTAGCCTGTGGAACTGGTTCTAA-3′

The synthetic T20 gene obtained from the commercial source was amplified and factor Xa cleavage site sequence was added to upstream to the T20 gene sequence and the whole sequence was amplified by PCR using gene specific primers to produce T20 amplified product. The forward primer 5′-CGGGGATCCATTGAGGGTCGCTACACC-3′ contains BamH I site and the reverse primer 5′-CGGGAATTCTCATTAGAACCAGTTCCA-3′ contains EcoR I site with the stop codon. PCR amplification was performed using the following cycles: Initial 94 °C for 5 min, 25 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 10 min with a final hold at 15 °C.

Construction of growth hormone-T20 (pGHT) vector

The full length synthetic human GH gene (GenBank: M36282.1) cloned in pUC vector, was obtained from GenScript USA Inc. (Piscataway, NJ), was used to amplify 186 bp N-terminal fusion fragment using the following primers.

Forward primer 5′-CGCATATGTTCCCAACTATTCCACTGAGT-3′

Reverse primer 5′-CGGGATCCAGGGGTCGGGATACTTTCAGAGAAACTCAA-3′.

The primers contain Nde I site in the forward primer and BamH I in the reverse primer. The following PCR amplification cycles were used for the amplification of the fragment (7 kDa): Initial 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and final extension of 72 °C for 10 min with a final hold at 10 °C. The Plasmid 'pET21a' and the amplified GH product were digested with Nde I and BamH I and were ligated by T4 DNA ligase to get recombinant plasmid 'pGH'. This pGH containing GH tag, under T7 promoter, was used to clone T20 gene. The amplified products of T20 and pGH were digested with BamH I and EcoR I and the products were purified by gel elution. The purified products (the amplified T20 product and pGH) were ligated by T4 DNA ligase to get another recombinant plasmid 'pGHT'. Sequence of pGHT, which has T20 gene and partial GH fusion tag sequence, was confirmed by Sanger's dideoxy method.

E. coli Rosetta strain was transformed with recombinant plasmid (pGHT). Transformed cells were grown in shake flask and the glycerol stocks were prepared and stored at −80 °C for further use.

Fermentation and harvesting

The seed culture was prepared in Luria–Bertani (LB) medium and cultured for 7–8 h at 35 °C on a rotary shaker. Fermentation was carried out in a Biostat-C fermentor (B. BRAUN, Germany) in fed-batch mode. Modified LB medium was used for the fermentation. The culture was maintained at 35 °C for a period of 5 h and the expression was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). The cells were harvested after 3 h of IPTG-induction. The pH was maintained between 7.0 and 7.2 with 50% ammonia solution. The rate of agitation was maintained ~600 rpm and the dissolved oxygen was maintained above 40% saturation. Sterilized 10% silicon based antifoam agent (Sigma–Aldrich) was used to suppress formation of foam. The samples were drawn every hour and were analyzed by SDS–PAGE for the protein expression. The harvested culture was centrifuged at 6600*g* for 10 min, the supernatant was discarded and the cells were collected as wet pellet.

Cell lysis and inclusion body (IB) purification

The wet cell pellet was suspended in 4 L of lysis buffer (50 mM Tris and 5 mM EDTA, pH 8.0). Cells were disrupted by passing the suspension through high pressure homogenizer (NIRO-SOAVI) twice at 900 bar pressure. After homogenization, the IB fraction from the cell lysate was collected by centrifugation at 8000*g* for 20 min. The crude IB was first washed with lysis buffer, then with lysis buffer containing 0.5% Triton X-100 and finally with lysis buffer containing 2 M urea. Traces of detergent and urea were removed by a final wash with 20 mM Tris (pH 7.5) buffer. After the final wash, the pure IBs were collected by centrifugation at 12,000*g* for 20 min.

Solubilization of inclusion bodies (IB)

Washed IBs obtained from earlier step were suspended in 1.5 L of solubilization buffer (20 mM Tris pH 7.5, 0.2% sodium dodecyl sulfate (SDS)) and incubated by stirring overnight at 4 °C on a magnetic stirrer. Subsequently, the solution was centrifuged at 12,000*g* for 30 min. The protein concentration in the supernatant was measured by Lowry method [13]. The supernatant was then diluted with 20 mM Tris buffer (pH 7.5) to adjust the SDS concentration to less than 0.1% and also the protein to ~5 mg/mL.