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EFFICIENT RECOMBINANT STRATEGY FOR THE PRODUCTION OF ENFUVIRTIDE IN *E.coli*

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ABSTRACT

Enfuvirtide (formerly known as T20) is a HIV fusion inhibitor, the first of its novel class of antiretroviral drugs, approved for HIV-positive people who have tried other currently available HIV drugs in the past and are unable to keep their viral loads at undetectable levels. It consists of 36 amino acids (~4.5 kDa) and binds to a key region of an HIV surface protein called gp41. T20 blocks HIV viral fusion by interfering with certain structural rearrangements within gp41 that are required for HIV to fuse and enter a host cell. Presently Enfuvirtide is being used as the last resort for HIV treatment though it is highly effective, because the production of synthetic T20 is difficult and expensive; hence we attempted the preparation of the same in recombinant method. The T20 peptide was fused with a 7 kDa N-terminal Growth Hormone (GH) tag in conjunction with enterokinase cleavage site and expressed in *E.coli* as inclusion bodies. Purified inclusion bodies were solubilized and cleaved with enterokinase. The digested fragments were purified through preparative reverse phase chromatography. The purity of rT20 was greater than 98.5%, determined by RP-HPLC and the yield was about 220-240 mg per liter harvest. Recombinant T20 peptide which was prepared under cGMP in a cost effective manner was found to be indistinguishable from the synthetic peptide (Fuzeon, Roche) by SDS-PAGE, mass spectroscopy and activity assay.

Keywords: cleavage with enterokinase; Enfuvirtide; fusion protein expression; HIV fusion inhibitor; Growth hormone tag; recombinant T20;

INTRODUCTION

Human Immunodeficiency Virus (HIV) affects specific cells of the immune system, called CD4 cells, or T cells and destroys these cells over time. This infection leads to Acquired Immuno Deficiency Syndrome (AIDS); a fatal syndrome leaves the victim susceptible to infections, malignancies, and neurological disorders. More than 25 million people have died of AIDS worldwide since the first cases were reported in 1981 and 35.3 million are currently suffering from HIV/AIDS [www.aids.gov, www.who.int]. Although there is no cure for AIDS, medications have been highly effective in fighting HIV and its complications. There are currently five different

"classes" of HIV drugs and most likely need to be used in combination. These include Nucleoside Reverse Transcriptase Inhibitors, Protease Inhibitors and Fusion or entry Inhibitors [www.fda.gov].

Entry inhibitors are a new family of antiretrovirals presently represented only by one drug, Enfuvirtide (T20) is the first fusion inhibitor to be approved by the Food and Drug Administration for the treatment of chronic human immunodeficiency virus (HIV) infection in adults and children 6 years and older [1,2]. Enfuvirtide targets HIV differently than currently available drugs by targeting multiple sites on gp41 and gp120 [3]. However, few clinical trials have determined that Enfuvirtide, worked best when it was combined with at least two other drugs that the virus was sensitive [4,5,6]. Many other compounds are in the process of clinical development and definitely will be part of the therapeutic armamentarium within the next few years. These compounds may prove beneficial for the growing number of HIV-infected individuals who have developed resistance to the currently available reverse transcriptase (RT) and protease inhibitors [7].

Enfuvirtide also called as fusion inhibitor that prevents the completion of the HIV fusion sequence and blocks virus' ability to infect healthy CD4 cells. It can reduce the amount of HIV count in the blood and increase the number of CD4 cells [2]. Enfuvirtide, different from other antiretroviral drugs by its extra-cellular action, operates by disrupting the HIV-1 molecular machinery at the final phase of fusion with the target cell, preventing healthy cells from becoming infected. Enfuvirtide interferes with the entry of HIV-1 into cells by inhibiting fusion of viral and cellular membranes. It binds to the first heptad-repeat (HR1) in the gp41 subunit of the viral envelope glycoprotein and prevents the conformational changes required for the fusion of viral and cellular membranes. Due to this HIV cannot bind with the surface of CD4 cells, thus preventing the virus from infecting healthy cells [1,7].

Synthetic Enfuvirtide (Fuzeon, Roche Pharmaceuticals, New Jersey) is a linear 36-amino acid synthetic peptide composed of naturally occurring L-amino acid residues with the N-terminus acetylated and the C-terminus is a carboxamide [8].Though Synthetic Enfuvirtide holds promise for HIV-positive patients who have taken (and failed) numerous HIV drugs in the past, it is being used only as a last resort, due to its high cost [9] and impurity levels. Hence we tried to prepare the same in recombinant method which is cheaper, safer, easier and rapid.

T20 peptide was tagged with N-termial Growth Hormone (GH, mol. wt. ~ 7 kDa) and expressed as a fusion protein as inclusion bodies (IBs) in *E.coli*. After preparation and partial purification of IBs, solubilization was done. Followed by this fusion protein was captured by anion exchange chromatography and cleaved with enterokinase to release C-Terminal rT20 peptide. The scope of this work is to prepare the intact rT20 peptide in economic conditions by using the recombinant bovine enterokinase for cleavage of enfuvirtide fusion protein instead of other cleavage agents such as Factor Xa, CNBr, trypsin. Enterokinase (E.C. No. 3.4.21.9) is a site-specific protease that recognizes and cleaves after the C-Terminal end of Lysine residue in the recognition sequence, DDDDK [10,11]. The rT20 peptide was further purified by preparative reverse phase chromatography. The purity and activity of rT20 was determined by RP-HPLC and HIV-1 p24 antigen capture assay kit respectively.

MATERIALS AND METHODS

Materials

T20 gene and PCR primers were synthesized by IDT Technologies (Coralville, Iowa, USA). Thermo cycler from Techne Ltd. (Cambridge, U.K.) was used for PCR amplification. Plasmid (pET21a) and *E.coli* host cells (Rosetta) were obtained from Novagen (Darmstadt, Germany).

BioStat-C Fermentor (15 L capacity) was procured from Sartorius, B. BRAUN, Germany. High pressure homogenizer (Model: NS100L Panada) from GEA NiroSoavi (Italy) was used for cell lysis. AKTA Purifier FPLC System, Q Sepharose 6 Fast Flow Anion Exchange media and HiScale 50/40 column were procured from GE Healthcare (Germany). Source-30™ Reversed Phase Chromatography media and Fine Line 35 column was purchased from GE Healthcare (Germany) for rT20 peptide purification. Recombinant bovine enterokinase was prepared in-house for cleavage of fusion protein. The Enfuvirtide standard 'Fuzeon' was purchased from Roche Pharmaceuticals (New Jersey). All other GR grade chemicals were procured from Merck Millipore, USA.

Methods

Cloning of T20 gene

The T20 nucleotide sequence (GeneBank Accession No AAA76666.1) was designed based on the known sequence of HR2 domain of gp41 glycoprotein (from 638-673 amino acid regions). The following complete monomer (108 bases) template was designed using the codon preference of the *E.coli.*

5'TACACCAGCCTGATCCACAGCCTGATCG
AAGAAAGCCAGAACCAGCAGGAAAAAAA
CGAACAGGAACTGCTGGAACTGGACAAAT
GGGCTAGCCTGTGGAACTGGTTCTAA -3'

The synthetic T20 gene was amplified and enterokinase cleavage site sequence was added upstream to the T20 gene sequence and the whole sequence was amplified by PCR using gene specific primers to produceT20 amplified product. The forward primer 5'-CGGGGATCCGATGACGAT GATAAATACACC-3' contains BamH I site and the reverse primer 5'-CGGGAA TTCTCATTAGAACCAGTTCCA-3' contains EcoR I site with the stop codon. PCR amplification was performed using the following cycles: Initial denaturation at 94^{o}C for 5 min, 25 cycles of 94^{o}C for 30 sec, 56^{o}C for 1 min, 72^{o}C for 1 min, and a final extension of 72^{o}C for 10 min with a final hold at 10^{o}C.

Construction of growth hormone-T20 (pGHET) 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 Forward primer 5'-CGCATATGTTCC CAACTATTCCACTGAGT-3'and Reverse primer 5'-CGGGATCCAGGGGTCGGG ATACTTTCAGAGAAACTCAA-3'.

The primers contain Nde I site in the forward primer and BamH I in the reverse primer. The PCR amplification cycles used were - Initial denaturation at 94^{o}C for 5 min; 30 cycles of 94^{o}C for 30 sec, 55^{o}C for 1 min, 72^{o}C for 1 min, and final extension of 72^{o}C for 10 min with a final hold at 10^{o}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 PCR amplified product 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 'pGHET'. Sequence of pGHET, which has T20 gene and partial GH fusion tag sequence, was confirmed by Sanger's dideoxy method. The recombinant pGHET plasmid was transformed into *E.coli*. Rosetta strain and the cells were grown in shake flask and the glycerol stocks were prepared and stored at -70^{o}C for further use.

Expression and Harvesting

One glycerol stock was inoculated into LB media containing 100 µg/mL ampicillin in 100 mL shake flask. The culture was incubated at 37^{0}C on orbital shaker at 200 rpm for 7-8 hours until the OD_{600} reached 1±0.1. Pilot-scale fermentation was

carried out (Biostat-C B. BRAUN, Germany, 15 L capacity) in fed batch mode. Modified TB media was taken into fermentor and sterilized. Then the seed culture was transferred into media and allowed to grow at 37^0C, at pH-7.0-7.2, for 6-8 hours. Soybean oil (Food grade from Sigma Aldrich) was used as anti-foaming agent. 1M phosphoric acid and 50% ammonia stock solutions were used for pH adjustment. Rate of agitation was 200-600 rpm and 30% dissolved oxygen level was maintained. 1 mM IPTG (from 1 M stock solution) was added to induce the expression when the OD_{600} reached to 35. Samples were collected in each hour for SDS-PAGE analysis. The culture was harvested after 3 hours of induction and centrifuged at 4000 rpm for 20 minutes at 4^0C. The cell pellet was collected and supernatant was discarded.

Cell Lysis and Purification of Inclusion Bodies(IBs)

The cell pellet was suspended in ice cold TE Buffer (Lysis Buffer-50 mM tris, 5 mM EDTA, pH-8.0), at 1:10 ratio. Then the cells were lysed with High Pressure Homogenizer at 950-1000 bar pressure, for 3 cycles at 4^0C, followed by centrifugation at 12000 rpm for 30 minutes at 4^0C.Inclusion bodies were collected aspellet. The pellet was washed with lysis buffer and further with lysis buffer containing 0.5% Triton X-100) and finally TE Buffer wash was given to remove residual Triton X-100.

Solubilization of GHET Fusion Protein

The Purified IB pellet was suspended in 50 mM tris, 6M Urea, pH-8.0, in 1:20 ratio and allowed for stirring at 2-8^0 C for 2 hrs. The solubilized sample was diluted with 50mMtris, pH-8.0, in 1:2 ratios prior to centrifugation at 12000 rpm for 30 min at 4^0 C. The supernatant was collected.

Purification of GHET fusion protein

The GHET fusion protein was purified through Q-Sepharose 6 Fast Flow medium packed in HiScale 50/40 Column. The column was equilibrated with 50 mM tris, 3 M Urea, pH-8.0 for 10 column volumes. Then the solubilization supernatant was loaded onto the column, followed by post load wash with equilibration buffer for 5 column volumes. The bound fusion protein was eluted with linear gradient using 200 mM NaCl in equilibration buffer.

Cleavage of GHET fusion protein with Enterokinase

The pure fractions were pooled together and diluted with 20mMtris-HCl, pH-8.0, at 1:4 ratio to reduce the urea concentration below 1 M. Recombinant bovine enterokinase (prepared in-house) was added at 1:50 (w/w) ratios. 2 mM $CaCl_2$ was added as inducer. The digestion mixture was incubated at 15^0C temperature, for 4 hours to release Enfuviritide peptide containing 36 amino acids.

Purification of rT20 peptide

The rT20 peptide was purified using Source 30 RPC media. The digested sample was loaded onto FineLine 35 column which was equilibrated with 0.2% TFA in Water, for 6 column volumes and post load wash done with equilibration buffer for 4 column volumes. 0.2% TFA in Acetonitrile solution was used as organic modifier to elute rT20 peptide in a linear gradient elution method. The elution fractions were analyzed on SDS-PAGE and the purified fractions were kept for lyophiziation for overnight. The lyophilized powder was reconstituted in WFI and analyzed by RP-HPLC.

Analysis of rT20 peptide

All the expression, Solubilization, purification samples and the lyophilized peptide was analyzed by SDS-PAGE in 15% gel and stained with coomassie brilliant blue R250. The purity of the final peptide was analyzed by RP-HPLC technique using Octadecylsilane column (4.6 mm x 150 mm, 5 µ bead size, Grace Vydac, USA). 0.1% TFA in WFI and 0.1% TFA in Acetonitrile were used as mobile phase A & B respectively with a linear gradient program of 0 to 100% B in 55 minutes, at 1 mL/min flow rate. The molecular weight of the rT20peptide was confirmed by MALDI-TOF mass spectrometry.

Activity of rT20 was estimated by using p24 antigen capture assay method and compared with its synthetic counterpart (Fuzeon, Roche). The assay method is based on the detection of HIV-1 p24 core antigen. 10^6 per mL Sup-T1 cells with 100% viability were selected in RPMI1640 medium containing 0.1% FBS in microtiter plates. Increasing concentrations of samples were added to wells. The cells were infected with HIV-193IN101 (Indian common HIV subtype C) at a concentration that is equivalent to 2 ng of p24 antigen per mL. The infected cells were incubated at 37^0C and 5% CO2. The cells were removed from the wells after 2 hrs of incubation by centrifugation at 350 g for 10min and the supernatant (free virus) was discarded. The cells were re-suspended in the freshmedium and incubated for 96 hours. On the fourth day of post infection, the supernatants were collectedfrom each well, mixed with equal volumes of Triton X-100, and assayed for p24 antigen as perthe p24 antigen capture assay kit (SAIC Frederick) protocol. The infection in the absence ofpeptide was considered to be 0% inhibition.

RESULTS & DISCUSSION

pGHET construction

The enterokinase-T20 gene amplification was performed from the synthetic template using gene specific forward and reverse primers. The N-terminal 186 base pair fragment of growth hormone encoding gene was amplified using the set of forward and reverse primers specific N-terminal part of Growth Hormone. Amplified N-terminal GH wasdigested with Nde1and BamH1 and ligated with digested pET21a(+) vector. The construct was named as pGH. The amplified enterokinase-T20 gene was inserted downstream to the N-terminal GH encoding sequence and the construct was named as pGHET (Figure 1). The sequencing results of cloned construct revealed that the construct was intact without any manipulation of N-terminal GH fusion partner, enterokinase linker and T20 gene.

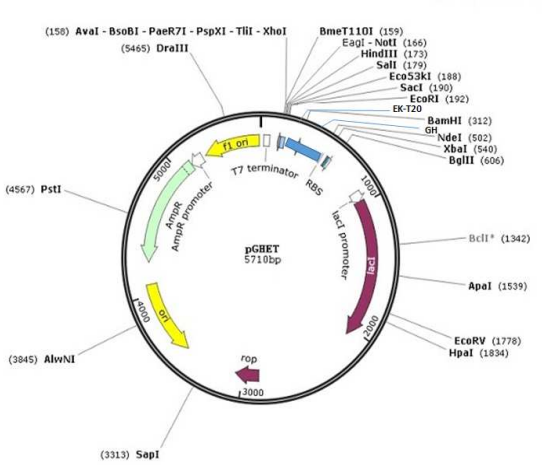


Figure 1: pGHET construct design

Fermentation

Fed batch fermentation was carried at 15 liter scale. High level expression of GHET was achieved up on induction with 1 mM IPTG. The cell density was enhanced by using modified TB media, under fed-batch conditions. Fed-batch fermentations take advantage of the high-protein-synthesizing capacity of an exponentially growing culture while minimizing the buildup of toxic by-products by keeping growth carbon source limited [12].The temperature was raised to 37^0C and dissolved oxygen concentration was reduced to 25%, during fermentation to form more IBs which obviously limited the loss of expressed peptide in soluble form. About900g cell mass was collected (at 60 g/L wet cell density) with not less than 15% expression (Figure 2).

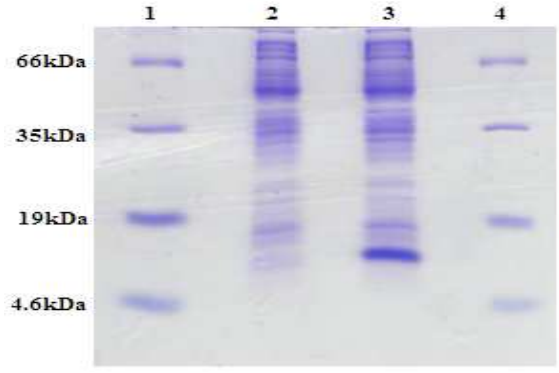


Figure 2: SDS-PAGE analysis of expression samples. lane 1: Protein molecular weight marker; lane 2: Expression sample before induction; lane 3: after three hours of induction; lane 4: Protein molecular weight marker.

Lysis &Purification of IBs

The cell lysis was carried out with high pressure homogenizer at 1000 bar pressure for 3 cycles to release intracellular insoluble protein. About 220 g of crude IBs were collected after centrifugation. IBs were washed with Triton X-100 to remove host cell proteins. Lysis and inclusion body washings were optimized to remove maximum contaminants in the centrifugation supernatant (Figure 3).

Solubilization of IBs and Purification of GHET fusion protein

Inclusion bodies were solubilized with 6 M urea. The concentration of urea was optimized in such a way that most of the HCPs to remain in insoluble form and to achieve maximum solubilzation of protein of interest. The purity of solubilization supernatant was about 60-70% (Figure 3) and concentration was about 9.5-10 mg/mL determined by Lowry method [13] (data not shown).

Solubilization sample was diluted with 50 mM tris-HCl, pH-8.0, prior to centrifugation, to reduce the concentration of fusion protein (which was required to purify through anion exchange chromatographic purification to avoid concentration overloading). The diluted solubilization supernatant was purified trough Q-Sepharose 6 Fast Flow media, packed in HiScale 50/40 column. Most of the HCPs were eliminated in flow-through. The purity of eluted fusion protein was more than 90% (Figure 4).

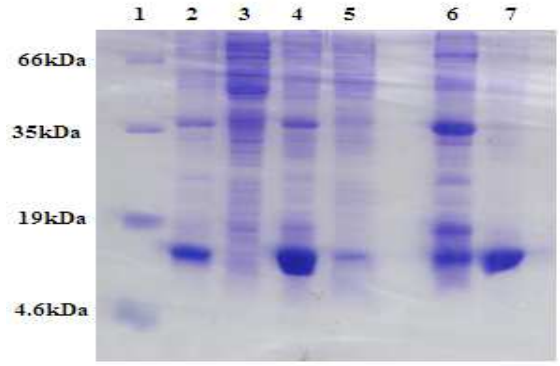


Figure 3*:* SDS-PAGE analysis of solubilization samples. lane 1: Protein molecular weight marker; lane 2: lysis pellet; lane 3: lysis supernatant; lane 4: IB wash pellet; lane 5: IB wash supernatant; lane 6: solubilization pellet; lane 7: solublization supernatant.

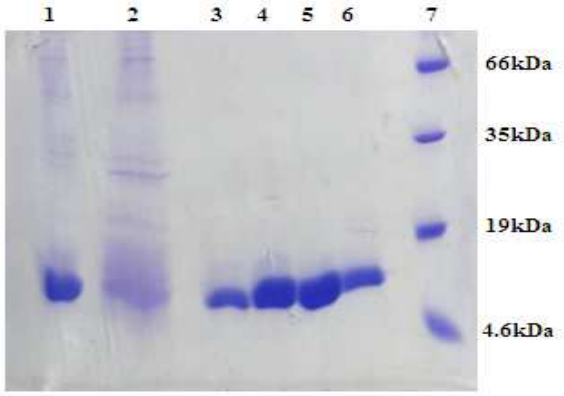


Figure 4: SDS-PAGE analysis of Q-Sepharose elute samples. lane 1: Q-Sepharoseload sample (solubilization supernatant); lane 2: flow-through; lane 3, 4, 5 and 6: elution fractions; lane 7: Protein molecular weight marker.

Cleavage of GHET fusion protein with Enterokinase

Enterokinase is a site-specific serine protease (E.C.3.4.21.9) that recognizes and cleaves after the C-terminal end of lysine residue of the GH tag (fusion partner) in the recognition sequence, DDDDK [10,11] and results in release of rT20 peptide containing 36 amino acids without any N-terminal modification.The cleavage of GHET fusion protein by enterokinase gave two fragments of GH tag (fusion partner ~7 kDa) and rT20 peptide (~4.5 kDa) (Figure 5).

Cleavage efficiency was increased by maintaining an optimum substrate (fusion protein) concentration of 1.5-3 mg/mL. Presence of 0.5-1 M urea and 25-50 mM NaCl, in digestion sample enhanced the cleavage efficiency. The function of urea in cleavage step is to ensure the free access of cleavage site to enzyme [14]. 2 mM $CaCl_2$ was added as inducer to maximize the cleavage of fusion protein [15].

Purification of rT20 peptide byPreparative Reverse Phase Chromatography

The enterokinase digested fragments were loaded onto Source 30RPC column. Initial 20% acetonitrile wash was given to remove loosely

bound impurities and the pure rT20 peptide, was eluted at around 40% Acetonitrile gradient (Figure 5) with a yield of 220-240 mg of peptide per liter harvest. GH tag was eluted at more than 55% acetonitrile. TFA was used as ion pairing agent to ensure hydrophobic interaction based separation. In order to enhance the selectivity there by resolution, the concentration of TFA has been increased to 0.2% in both mobile phases [16].

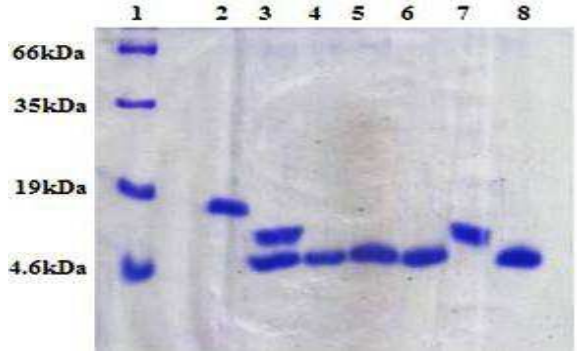


Figure 5*:* SDS-PAGE analysis of cleavage and RP purified samples.
lane 1: Protein molecular weight marker; lane 2: uncut/fusion protein; lane 3: enterokinase digested fragments (GH tag and T20 peptide) and the same sample is loaded to RP Column; lane 4, 5 and 6: elution fractions 1, 2, and 3 respectively showing pure rT20 peptide; lane 7: elution fraction 4 (GH tag); lane 8: Reference peptide (Fuzeon).

Characterization of rT20 peptide

SDS-PAGE

The final peptide was analyzed on SDS-PAGE along with reference standard. There were no detectable impurities found in the test sample.The molecular weight of both test and reference was same and observed nearby 4.6 kDa (Figure 6).

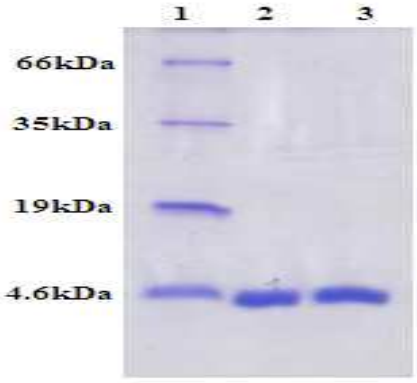
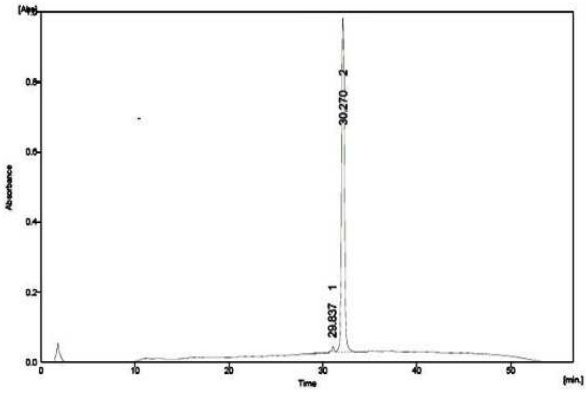


Figure 6*:* SDS-PAGE analysis of rT20 peptide. lane 1: Protein molecular weight marker; lane 2: rT20 peptide prepared in-house; lane 3:Reference standard peptide (Fuzeon).

RP-HPLC Analysis

The purity of the rT20 peptide was determined by RP-HPLC and was more than 98.5%. The retention time of peptide was 30.27 minutes (Figure 7).



Result Table

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	29.837	185.227	0.052	1.348	2.206e-02	0.01
2	30.270	13550.967	234.244	98.652	100.0	0.71
	Total	13736.195	234.296	100.000	100.0	

Figure 7*:* RP-HPLC analysis of rT20 peptide

Biological activity of rT20 peptide

Recombinant T20 peptide and synthetic T20 peptide in different concentrations (from 1 ng to 10 mcg) were analyzed for biological activity by using p24 antigen capture assay method. The calculated p24 antigen inhibition percentage of rT20 peptide was similar to the synthetic T20 peptide (Fuzeon) from Roche Pharmaceuticals, New Jersey (Figure 8).

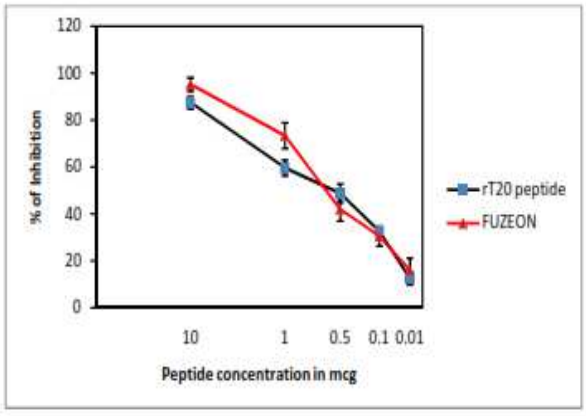


Figure 8: Activity comparison between rT20 and Fuzeon

CONCLUSION

This expression and purification strategy allows for large-scale production of Enfuvirtide, which retain the activity similar to the peptide produced by chemical synthesis method. Purification was achieved in three steps, which are easily scalable for industrial application. In summary, we present efficient recombinant strategy which will enable cost-effective production of Enfuvirtide under cGMP conditions.

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