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CHEMISTRY DEPARTMENT

Design and production of a potential HIV-entry inhibitor

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ABSTRACT

Human immunodeficiency virus (HIV) infection is a major health problem worldwide. Although it not curable, after the discovery of the antiretroviral drugs, it has become treatable and the prognosis of the patients has improved. However, the high toxicity of these drugs and the development of resistance have made necessary to find a different way to combat the disease. Recently, a new kind of HIV inhibitor that blocks the fusion of the protein and the viral membranes, has been discovered. Among these, Enfuvirtide (Fuzeon) has been the first entry inhibitor to be accepted as a drug for human use.

The present work proposes the production of a dimer of Enfuvirtide which is thought to become a stronger HIV entry inhibitor. Due to the high cost of Fuzeon production, it will be obtained as part of a fusion protein expressed in bacterial cells and cleaved in order to remove the peptide.

The preliminary steps previous to the expression of the fusion protein are shown.

1. INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) was recognized in 1981, and the first human immunodeficiency virus (HIV) was isolated 2 years later^{xxvii}. Since then, HIV has spread worldwide, with an estimated 39.4 million infected people by the end of 2004^{xxviii}. HIV is a lentivirus that is predominantly transmitted by sexual contact. It crosses the mucosal epithelium and infects cells expressing the CD4 receptor, causing gradual reduction of CD4⁺T cells, which leads to an increased sensitivity to opportunistic and chronic infections and to oncogenesis.

Currently, there have been discovered more than 20 anti-HIV drugs. These antiretroviral drugs (ARV) are categorized according to their mode of action. Nucleoside reverse transcriptase inhibitors (NRTI) block the action of transcriptase reverse (TR) which is responsible for transcribing the HIV-1 RNA genome to DNA, non-nucleoside reverse transcriptase inhibitors (NNRTI) do not bind to the active site of TR but to a different site and cause conformational changes that reduce the enzyme activity, and protease inhibitors (PI) that bind the protease active site and inhibit the cleavage of viral proteins^{xxix}.

These ARV drugs are administered in combination (cocktails of 2 or 3 types) and this highly active antiretroviral therapy (HAART) has improved the prognosis for HIV patients. However, toxicity and resistance to some or all of the ARV drugs have been found among patients.

Recently, a new generation of inhibitors has been discovered. These HIV entry inhibitors inhibit viral cell entry. The first drug approved of this kind was Enfuvirtide (Fuzeon, formerly known as T-20 and DP-178)^{xxx}. Among its advantages, this drug has activity against multidrug resistant virus and limited side effects. In order to understand the mode of action of Enfuvirtide is important to address the HIV entry pathway.

HIV fusion

HIV is an envelope virus that has spikes on its surface consisting of a protein complex that comprises a cell-surface attachment glycoprotein, gp120^{xxx}, and a membrane spanning protein, gp41. These are non-covalently linked and assemble into an oligomer (trimer). Viral entry begins with the binding of gp120 to CD4 receptors on the surface of lymphocytes. This step is followed by the binding to co-receptors (CXCR4 or CCR5) that causes a conformational change on gp120 and exposes the transmembrane gp41^{xxxii}. gp41 contains two heptad repeat regions: HR1 (proximal to the N terminus) and HR2 (proximal to the C terminus)^{xxxiii}, a N-terminal fusion peptide (FP) and a transmembrane region that anchors the protein to the viral membrane. The FP inserts into the host cell membrane, whereas the HR2 regions zips around a preformed trimer of HR1 regions in an antiparallel fashion to form a six-helix bundle that attracts the viral and the membranes together for fusion^{3,7,xxxiv}. Because the fusion process does not occur immediately (formation of a pre-hairpin intermediate step is believed to occur), this process can be inhibited.

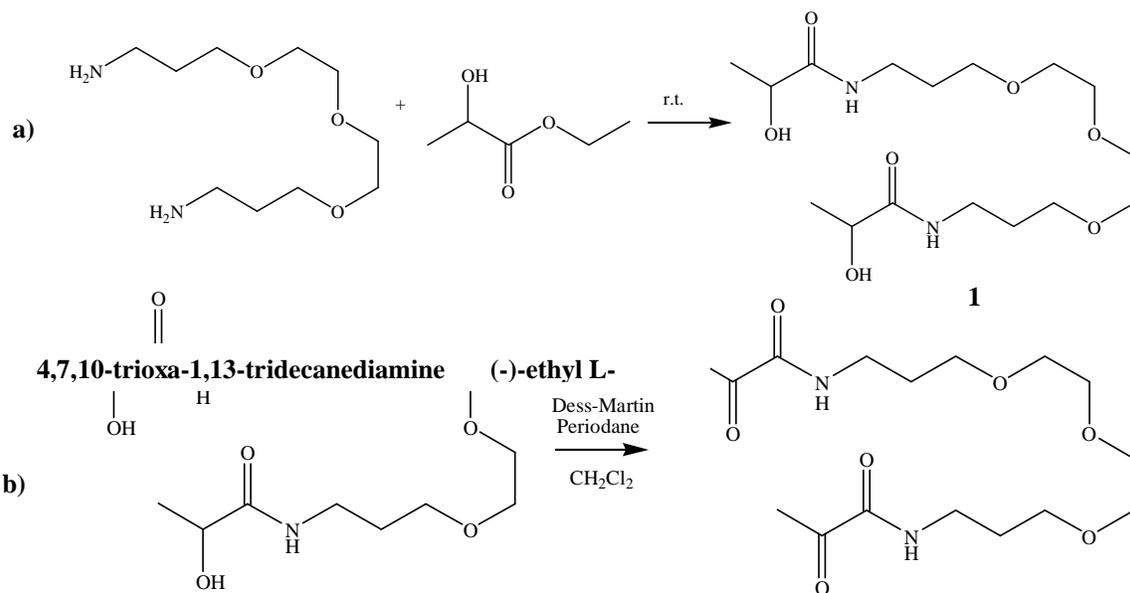
Enfurvitide (Fuzeon): An HIV entry inhibitor

observe an antiviral effect in humans. Cheaper methods of production of the drug are necessary in order to satisfy its demands.

Design of a potential HIV inhibitor: dimeric Enfuvirtide

Enfuvirtide mimics the C-terminal sequence of gp41, and binds to the N-terminal sequence (HR1 domain) inhibiting the fusion between the host and the viral membrane. Considering that the N-peptide trimeric coiled coil contains three grooves that can be bound by C-peptides, in this work, we propose the production of a dimer of Enfuvirtide, which is thought to bind to two of the grooves of the HR1 domain. This molecule is believed to become a better inhibitor than the monomer itself.

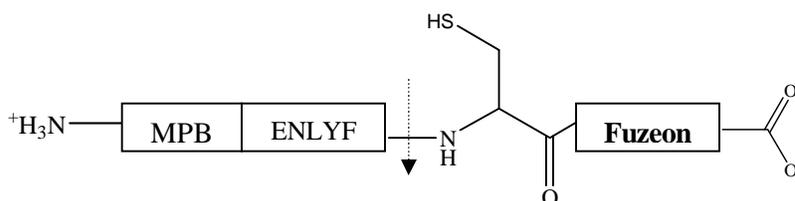
In the dimer the peptides will be joined by a linker that contains amide and ether as functional groups, and a terminal ketone. Polyether linkers are soluble in water. The linker will be synthesized by the route shown in **Scheme 1**.

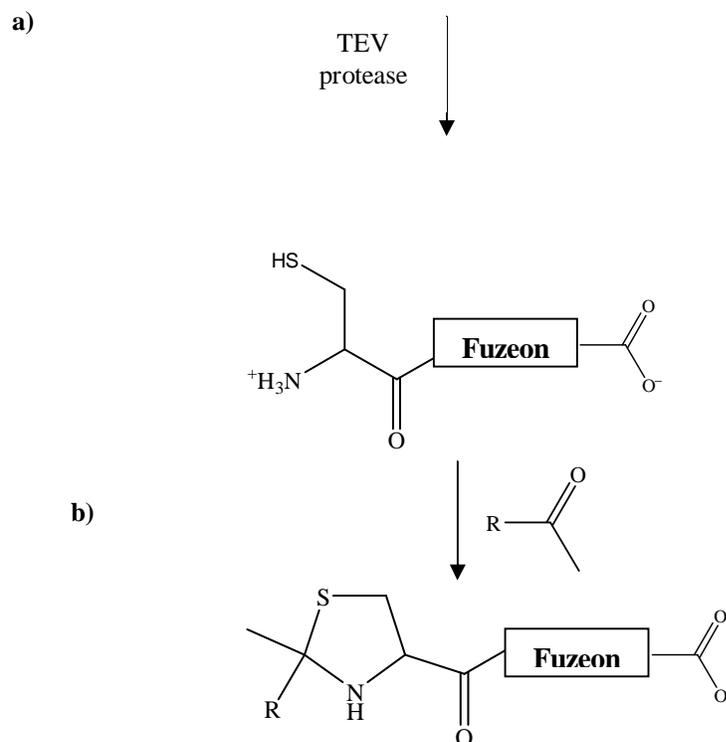


Scheme 1. Synthetic route proposed for the production of the linker. a) Formation of the diamide linker, 1^{xxxvi}. b) Formation of the terminal ketone linker, 2.

Production of Enfuvirtide by bacterial expression

Because the synthesis of Enfuvirtide is highly costly, we propose to obtain the peptide as part of a fusion protein which will be produced by bacterial expression^{xxxvii}. The protein partner is necessary because it serves to stabilize the peptide during expression of the fusion protein in bacteria. Maltose-binding protein (MBP) will be used because it has been proven to enhance the solubility of proteins expressed in *E.coli*^{xxxviii}. After expression, Enfuvirtide will be cleaved from the fusion protein using tobacco etch virus NIa protease (TEV protease) (**Scheme 2, a**). TEV protease recognize a seven amino acid site and cleaves between a glutamine and a serine or glycine. However, it has been proven that it can cleave between a glutamine and a cysteine without losing substantial activity¹³. The formation of a N-terminal cysteine will be necessary in order to ligate chemoselectively Fuzeon and the synthesized linker (**2**) (**Scheme 2, b**)^{xxxix}.





Scheme 2. a) Cleavage of the fusion protein by TEV protease. b) Chemoselective ligation between the N-terminal cysteine of Fuzeon and the linker. Formation a of thiazolidine linker.

2. MATERIALS AND METHODS

2.1. General

Plasmids pET28a(+), pMAL-c2x and *E.coli* strain TOP10F': *F'* [*lacI^q*, *Tn10(Tet^R) mcrA* Δ *Imrr-hsdRMS-mcrBC*) ϕ 80 *lacZ* Δ *M15* Δ *lacX74* *deoR* *recA1* *araD139* Δ (*ara-leu*)7679 *galU* *galK* *rpsL(Str^R)* *endA1* *nupG*] were purchased from New England BioLabs.

Primers 5'-Eco-TEV-Fuz (GCGCCGGCCcatatgTACACCTCCCTGATCCACTCC), 3'-HindIII-Fuz (GCGCGCgaattcttaGAACCAGTTCCACAGGGAAGC), 5'-Eco-TEV-Fuz (5'-GGC CCG GAA TTC GGA GGT GAA AAC CTG TAT TTT CAG TGC GGT TAC ACC TCC CTG-3') and 3'-HindIII-Fuz (5'-GCG CGC AAG CTT TTA GAA CCA GTT CCA CAG GGA AGC-3') were purchased from MWG Biotech AG.

Enzymes *Taq* DNA Polymerase, T4 DNA Ligase, restriction endonucleases *EcoRI* and *HindIII*, and CIP (calf intestinal alkaline phosphatase) were purchased from New England BioLabs.

4,7,10-trioxa-1,13-tridecanediamine and (-)-ethyl L-lactate were purchased from Sigma-Aldrich. The reaction was followed by thin layer chromatography (TLC) using silica gel 60 F₂₅₄.

Growth medium

1 L of Luria Bertani (LB) medium pH 7 was prepared by mixing 10 g peptone, 5 g yeast extract and 10 g NaCl with deionized water.

Culture conditions

E.coli cells were grown in 5 mL LB medium containing 100 µg/mL ampicilin and shaking at 37 °C overnight.

DNA purification conditions

After culturing *E.coli* cells the plasmid DNA was purified using Quigen Q1 Aprep Spin Miniprep Kit (250) following the protocol: Plasmid DNA purification using the Q1 Aprep Spin Miniprep Kit and a Microcentrifuge.

After fragment digestion the DNA was purified using Quigen QIAquick PCR purification kit (250) following the protocol: QIAquick PCR purification using a microcentrifuge.

PCR reactions conditions

10x Thermo Pol Buffer B9004S, 200 µM dNTPs, 1 µM of each primer, 2 pg DNA plasmid, 0.5 µL *Taq* DNA Polymerase and milliQ water were mixed in a PCR tube. Controls were run using water instead of DNA plasmid. After an initial denaturation step of 3 min at 94 °C, 33 cycles of 45 s at 93 °C, 30 s at 55 °C and 2 min at 72 °C were performed. A final extension step of 10 min at 72 °C completed the reaction.

DNA concentration analysis

75 μL of 15x dilution solutions were used to determine DNA double strand concentrations at 280 nm using DU 530, Life Science UV/Vis Spectrophotometer, Beckman Coulter.

Tris-glycine SDS-polyacrylamide gels

17% acrylamide gel was prepared by mixing 2.5 mL of 1.5 M Tris-HCl pH 8.8, 0.05 mL of 20% SDS and 5.7 mL of a 30% acrylamide solution. The polymerization was initiated by the addition of 160 μL of 10% APS (ammonium persulfate) and 6 μL of TEMED (N,N,N',N'-tetramethylethylenediamine).

2 mL stacking gel was prepared by mixing 1.4 mL of water, 0.33 mL of 30% acrylamide solution, 0.25 mL of 1 M Tris-HCl pH 6.8 and 0.02 mL of 10% SDS. The polymerization was initiated by the addition of 0.02 mL of 10% APS and 0.002 mL of TEMED.

Agarose gels

1% and 2% agarose gels were prepared dissolving 0.6 g and 1.2 g agarose, respectively, in 60 mL 1x TAE buffer (40 mM Tris-HCl, 1mM EDTA) and 6 μL of a 1 mg/mL ethidium bromide. The gels were resolved using UV Transilluminator UVP.

Agarose gel extraction conditions

The gels were treated using the Quigen QIAEXII gel extraction kit (150) following the protocol QIAEXII agarose gel extraction.

Single digestion reaction conditions

Single digestions were carried out with 10 μL total volume using 1 μL 10x *EcoRI* buffer, 0.2 μL restriction enzyme, 8.6 μL pMAL-c2x plasmid DNA and milliQ water, for two hours at 37 °C.

Double digestion reaction conditions

Digestions of plasmid DNA were carried out in 50 μL total volume, using 5 μL 10x *EcoRI* buffer, 1 μL *HindIII*, 1 μL *EcoRI* and 43 μL pMAL-c2x plasmid DNA, for two hours at 37 °C. After this period of time 1 μL CIP was added. The enzymes were heat inactivated at 65 °C. The DNA plasmid was purified running a 1% agarose gel.

Digestions of fragment DNA were carried out in 20 μL total volume, using 16 μL Fuzeon DNA, 2 μL 10x *EcoRI* Buffer, 1 μL *HindIII* and 1 μL *EcoRI*. The DNA was purified running a 2% agarose gel.

Ligation reaction conditions

For 20 μL total volume, 2 μL 10x T4 DNA ligase buffer, 1 μL T4 DNA ligase, 7 μL pMAL-c2x plasmid DNA, the desire volume of Fuzeon DNA and milliQ water were mixed. The reaction was left at room temperature for two hours. Controls were made without insert.

Transformation and electroporation conditions

70 μL *E. coli* TOP10F' cells extract and 1 μL of the ligase mixture were transformed using an *E. coli* MicroPulser electroporator (BioRAD) at the field strength of 1.8 kV/cm and pulse duration of 3 ms, then incubated for 1 hour in 800 μL LB medium. Finally, the cells were planted onto dishes containing LB medium and 100 $\mu\text{g}/\text{mL}$ ampicilin.

2.2. Cloning of enfurvitide (Fuzeon) into pET-28a(+)

The gene encoding for Fuzeon was amplified using pET-28a(+) in which was previously inserted by Theodore Gries in the Tolbert Lab. A 25 μL reaction was run following the PCR reaction conditions. Fuzeon fragment was generated by using 5' primer: 5'-Met-Fuz-NdeI and 3' primer: 3'-Fuz-St-EcoRI. The presence of the Fuzeon DNA was determined using a 2% agarose gel.

2.3. Construction of the Fusion Plasmid

In order to insert Fuzeon DNA into the polylinker site of pMAL-c2x, it was necessary to clone it using different primers. The PCR reaction was run using 5' primer: 5'-Eco-TEV-Fuz and 3' primer: 3'-HindIII-Fuz. Analysis and purification of Fuzeon DNA was made using a 2 % agarose gel.

Plasmid pMAL-c2x and Fuzeon DNA were cut using *EcoRI* and *HindIII* restriction enzymes, ligation was done using T4 DNA Ligase at two different volumes of insert (2 and 7 μL), followed by transformation of *E. coli* TOP10F'.

2.4. TEV Protease cleavage

A solution of 200 μL $2.77 \times 10^{-7} \text{ M}^{*,x1}$ TEV protease (5 mM DTT, 200 mM NaCl, 40% glycerol) was placed into a 1000 MWCO dialysis bag. The bag was placed into 1 L 25 mM potassium phosphate pH 7.9 and left for three hours at room temperature. After this period of time, 400 μL of 5 mg/mL GPRT-C37-H6 fusion protein, prepared by Professor Tom Tolbert was added to the dialysis bag. The bag was placed into 1L 25 mM potassium phosphate pH 7.8, and the cleavage reaction was initiated by adding β -mercaptoethanol to a final concentration of 1 mM. The reaction was gently stirred and incubated at room temperature. It was monitored after 30 min, 1, 2 and 17 hours by running a 17 % acrylamide gel under denaturing conditions.

2.5. Synthesis of the diamide linker (1)

4,7,10-trioxa-1,13-tridecanediamine (0.16 mL, 9×10^{-4} mol) was added under inert atmosphere to (-)-ethyl L-lactate (1 mL, 4.5×10^{-3} mol, 5 equiv). The reaction mixture was first left at room temperature, then heated at 65 °C and finally at 105 °C until the starting material was gone. Reaction duration: 5 days. The reaction was followed using $\text{CH}_3\text{CH}_2\text{OCOCH}_3:\text{CH}_3\text{OH}/9:1$.

* TEV protease concentration was determined theoretically using its absorbance of 0.009 at $\lambda(280)$ following reference 13.

3. RESULTS AND DISCUSSION

3.1. Cloning of enfurvitide (Fuzeon) into pET-28a(+)

The gel obtained showed the formation of Fuzeon DNA (**Figure 2**). With this result it was concluded that the desired DNA fragment was successfully inserted into pET-28a(+) plasmid.

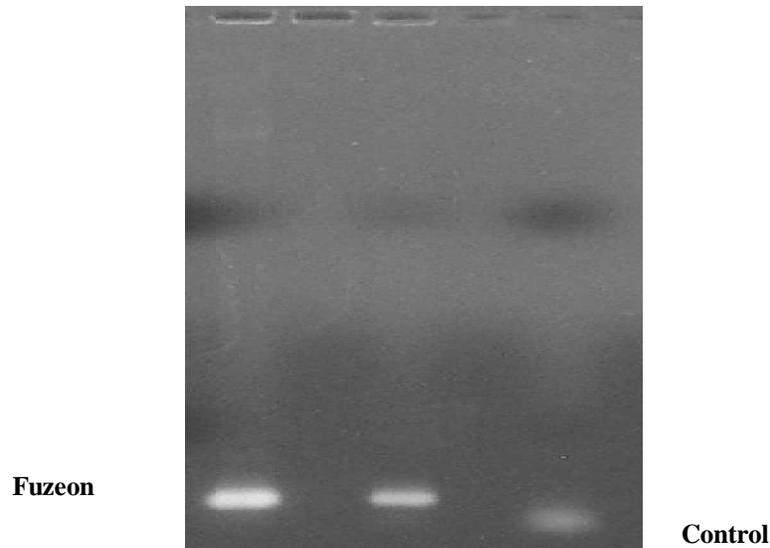


Figure 2. Fuzeon DNA from pET-28a(+) plasmid.

3.2. Construction of the Fusion Plasmid

The analytical gel run after the PCR reaction was analyzed at different moments to confirm that the Fuzeon DNA was running at a different Rf than the control. From this study it was believed that the primers were able to anneal between them during the PCR reaction in the absent of a vector, since the control run at a different Rf than the original primers (**Figure 3**). The purification of the insert gave a concentration of 43.828 µg/mL DNA.

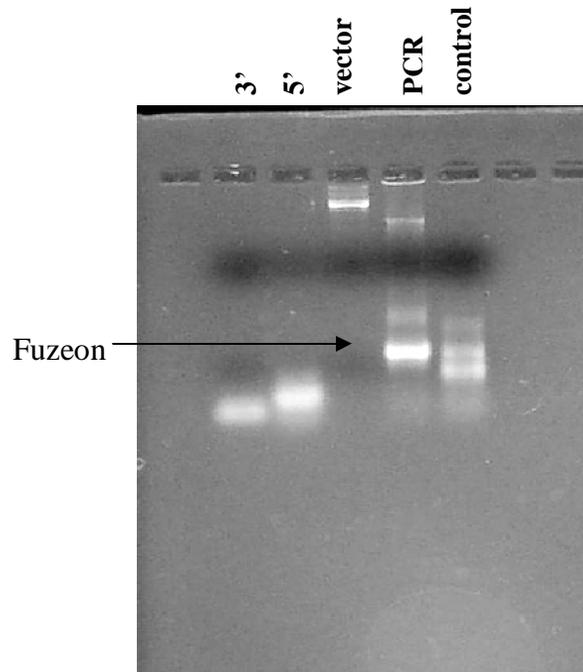


Figure 3. PCR reaction analysis.

Single digestions of the plasmid using *EcoRI* and *HindIII* were analyzed after 2 hours to confirm the activity of the restriction enzymes. The 2% agarose gel showed that both the enzymes were capable to cut the plasmid (**Figure 4**). The digested plasmid was successfully purified running a 1% agarose gel which gave two solutions of 50.966 $\mu\text{g/mL}$ and 29.286 $\mu\text{g/mL}$. The fragment was also digested, the concentration of the DNA obtained was 7.43 $\mu\text{g/mL}$.

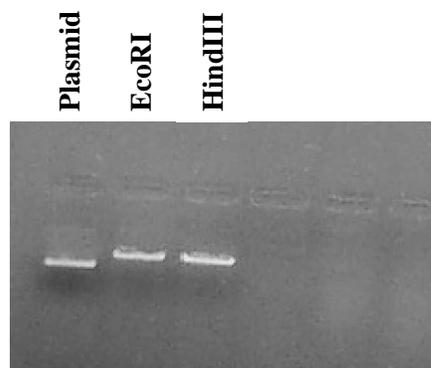


Figure 4. Single digestion of plasmid pMAL-c2x.

After ligation, transformation made with the ligation mixture of equal volumes of insert and plasmid did not produced colonies containing the desired plasmid. An appreciable number of colonies grew in the absence of insert and a few with a small volume of it. These results reflected that the plasmid could closed in the absent of insert and that its presence inhibited the plasmid to circularize. If the plasmid could circularize it could mean that CIP could not cut the phosphate groups leaving the possibility to the ligase to close the vector. However, although the activity of each restriction enzyme was confirmed, there still the possibility that one of them is inhibiting the activity of the other. It is also important to note that the insert was purified using the PCR purification kit from Quigen instead of using a gel, so there is a chance that the fragment was not appropriately cleaned.

3.3 TEV Protease cleavage

The acrylimide gel showed that the cleavage did not finish after 17 hours and a great amount of the fusion protein was left. It was believed that the dialysis made to the TEV protease could have decreased its activity.

A second reaction was set up without dialysis of TEV protease and following the procedure previously mentioned. The reaction was monitored after 30 min, 1, 2, 4 and 19 hours by running a 17 % acrylimide gel under denaturing conditions (**Figure 5**).

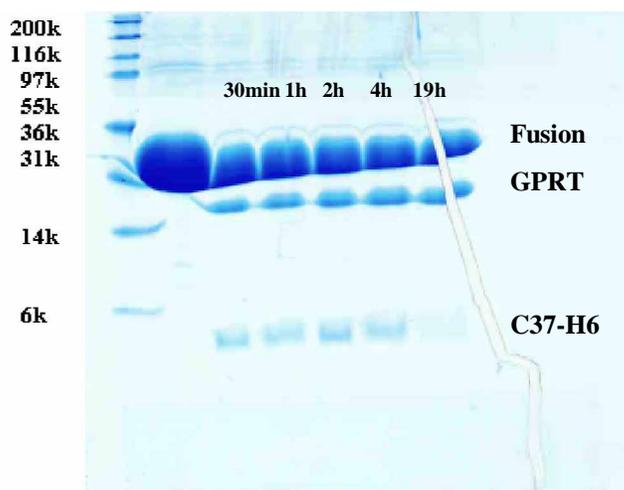


Figure 5. Cleavage of GPRT-C37-H6 fusion protein by TEV protease

The gel showed that the protein cleaved more fusion protein than in the previous study but it did not take the reaction to completion.

The poor activity obtained could have been caused by using a small quantity of the TEV protease or because this protein had a low specific activity.

3.4. Synthesis of the diamide linker (1)

It was found that in order to get the reaction to completion, it has to be heated at 105 °C, in which also the distillation of the ethanol formed is favored. Flash chromatography will be necessary to separate the desired product from what is believed to be the monoamide product.

4. CONCLUSIONS

The pET28a(+) contains the DNA sequence that will express Fuzeon and it can be used to clone this fragment in order to insert it in the plasmid desired, in this case pMAL-c2x that contains the sequence for the protein partner MPB.

The purification method used to purified Fuzeon DNA after its double digestion gave a small concentration of DNA, so a different method, like gel purification, should be tried in order to improve the yield.

Colonies containing the plasmid desired were not obtained. pMAL-c2x circularize in the absent of insert and a few colonies are obtained in its presence.

The TEV protease used in this study can not be used to cleave the fusion protein desired (MPB-Fuzeon). It will be necessary to express new protease with a high specific activity.

The synthesis of the diamide linker should be run at 105 °C in order to get into completion. The proposed synthesis seems to give the monoamide linker as a byproduct and flash chromatography is required to purify the product.

5. FUTURE WORK

The digestion of the plasmid will be made by adding one by one the restriction enzymes, with heat inactivation after 1 hour of reaction of one and before the addition of the second. This process will assure that both the enzymes will cut the plasmid. If the desired colonies are still not obtained, there will be a big probability that CIP is the cause of the problem.

Purification of the DNA fragment after digestion will be achieved by gel purification which is believed to achieve better results.

The synthesis of the diamide linker will be run at 105 °C. If a poor yield is obtained after flash purification a second reaction will be used to obtain the diamide linker. The alternative reaction uses lithium aluminium hydride (LiAlH_4) to deprotonate the nitrogens of 4,7,10-trioxa-1,13-tridecanediamine, making it a better nucleophile to attack the carbonyl in the (-)-ethyl L-lactate^{xli}.

6. REFERENCES

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