CONTROLLED IN *VIVO* PROCESSING OF scFv-MBP FUSION PROTEIN: TOWARDS HIGH LEVEL PRODUCTION OF SOLUBLE AND FUNCTIONAL scFv- BASED BIOPHARMACEUTICAL IN BACTERIAL SYSTEM

By

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DECLARATION

I declare that this dissertation records and results are performed by me, and it has not been submitted previously for a higher degree in any university.

Aly Atef Aly Shoun,

June, 2015

DEDICATION

I dedicate this work to my family and my colleagues in Dr.Ali's laboratory.

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LIST OF ABBREVIATIONS

^{0}C	Degree centigrade
2x	2 times
4x	4 times
6x	6 times
AIDS	Acquired Immunodeficiency Syndrome
Amp	Ampicillin
Amp^{R}	Ampicillin resistance
APS	ammonium persulfate
Bp	base pair
C	Capsid
CBB	Coomassie Brilliant Blue
Cm	Centimeter
cm3	Centimeter Cube
Da	Dalton
DNA	deoxyribonucleic acid
DNase	deoxynucleosides triphosphate
dNTP	deoxyribonucleoside-5´-triphosphate
DTT	Dithiothreitrol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay

Fab	antigen binding fragment
Fc	cristalline fragment
Fv	variable fragment
G	Gram
HCl	Hydrochloric acid
HIV	Human Immune deficiency Virus
IgG	immunoglobulin G
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Kb	kilo base
KOH	Potassium Hydroxide
LB	Luria-Bertani
SB	Super Broth
TB	Terrific Broth
min	Minute
Ml	milli liter
mm	milli meter
Mm	Micrometer
MW	molecular weight
NaOH	Sodium Hydroxide
NEB	New England Biolab
OD600	ptical density = absorbance at 600 nm
PBS	phosphate buffer with sodium chloride
PCR	polymerase chain reaction

PEG	polyethylenglycol
pH	Potential hydrogen
RNA	ribonucleic Acid
Rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcriptase PCR
S	Second
scFv	single-chain variable fragment
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE	Tris/acetate/EDTA buffer
TAE	Tris/acetate/EDTA(buffer)
Taq	Thermus aquanticus
TCA	trichloroacetic acid
TE 50	M Tris/HCl, 1 mM EDTA, pH 8.0
TEMED	N,N,N´,N´-tetramethyl-ethylendiamine
Tris	tris-(hydroxymethyl)-aminomethane
UV	Ultraviolet
UV/VIS	ultraviolet-visible
V	Volume
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organization

Controlled in vivo processing of scFv-MBP fusion proteins: Towards high level production of soluble and functional scFv-based biopharmaceuticals in bacterial system

ABSTRACT

In the past two decades, the bacterial expression of antibody fragments has shown great dependence in the rapid expansion and a key success in antibody engineering. Bacterial expression and antibody engineering provide a suitable means of creating antigen binding fragments for isolation, evaluation and production of antibody. In this study we succeeded to design specific primer for anti-HIV1-CA scFv, which was used in PCR amplification of anti-HIV1-CA scFv, MBP and vector pMXB10Hp24-6His. Subsequently, restriction enzyme analysis has been done using Nde1-R/Not1-F enzymes then the restricted insert and vector were ligated. The constructed plasmid, pMXB10-MBP-TEV-scFv46 was transferred into E.Coli DH5-α competent cells. Numerous positive clones were presented on the transformed plate and clones were randomly selected to be confirmed by colony PCR which showed the band for pMXB10-MBP-TEV-scFv46 clearly. The plasmid DNA was harvested, purified then transformed into NiCo21 (DE3), SHuffle (C3029) as competent cells for protein expression. The colony PCR was performed in order to confirm the presence of pMXB10-MBP-TEV-scFv46 clones in the transformant. Starter cultures were prepared in order to optimize expression of the protein of interest; (NiCo21-TEVEco and SHuffle-TEVEco) competent cells were used. Indeed, the expression of MBP-scFv-46 using

NiCo21-TEVEco as competent cell was mainly present in the insoluble fraction. Immunoblot analysis with the anti-6His antibody revealed that none of MBP-scFv-46 proteins was present in the soluble fraction. However, Almost 90%-95% of MBP-scFv fusion protein has been cleaved by TEV protease using SHuffle-TEVEco. Immunoblot analysis also showed that almost 40% of scFv was present in the soluble fraction, which mean that we succeeded to develop novel system in order to achieve Controlled *in vivo* processing of MBP-scFv fusion proteins: Towards high level production of soluble scFv from *E.Coli*.

Kawalan dalam pemprosesan *vivo* scFv-MBP protein gabungan: Ke arah pengeluaran yang tinggi biofarmaseutikal berasaskan scFv larut dan berfungsi dalam sistem bakteria

ABSTRAK

Dalam dua dekad yang lalu, ungkapan bakteria serpihan antibodi telah ditunjukkan pergantungan yang besar dalam perkembangan pesat dan kejayaan utama dalam bidang kejuruteraan antibodi. Ungkapan bakteria dan kejuruteraan antibodi menyediakan satu cara yang sesuai mewujudkan antigen mengikat serpihan untuk pengasingan, penilaian dan pengeluaran antibodi. Dalam kajian ini, kami berjaya untuk mereka bentuk buku asas tertentu untuk anti-HIV1-CA scFv, yang telah digunakan dalam PCR penguatan anti-HIV1-CA scFv, MBP dan pMXB10Hp24-6His vektor. Selepas itu, analisis enzim sekatan telah dilakukan dengan menggunakan Nde1-R / Not1-F enzim kemudian memasukkan dan vektor terhad telah ligated. The plasmid dibina, pMXB10-MBP-karena justru-scFv46 telah dipindahkan ke E.Coli DH5-α sel berwibawa. Banyak klon positif telah dibentangkan di atas pinggan yang berubah dan klon telah dipilih secara rawak untuk mengesahkan oleh koloni PCR yang menunjukkan dengan jelas band untuk pMXB10-MBP-karena justru-scFv46.

The plasmid DNA telah dituai, disucikan kemudian berubah menjadi NiCo21 (DE3), Shuffle (C3029) sel-sel yang mempunyai kebolehan untuk ungkapan protein. Koloni PCR telah dilakukan untuk mengesahkan kehadiran klon pMXB10-MBP-karena justru-scFv46 dalam transformant itu. Kultur pemula telah disediakan untuk mengoptimumkan ungkapan protein kepentingan; (NiCo21-TEVEco dan SHUFFLE-TEVEco) sel-sel yang berwibawa

telah digunakan. Menariknya, NiCo21-TEVEco menunjukkan Overexpressed MBP-scFv-46 protein Hgabungan adalah terutamanya hadir dalam pecahan tidak larut. Analisis Immunoblot dengan antibodi anti-6His mendedahkan bahawa tiada MBP-scFv-46 protein hadir dalam pecahan larut; Walau bagaimanapun, hampir 90% -95% daripada protein gabungan MBP-scFv telah melekang oleh protease karena justru menggunakan SHUFFLE-TEVEco. Analisis Immunoblot juga menunjukkan bahawa hampir 40% daripada scFv hadir dalam pecahan larut, yang bermakna kita berjaya untuk membangunkan sistem baru bagi mencapai Kawalan dalam pemprosesan *vivo* MBP-scFv protein gabungan: Ke arah pengeluaran yang tinggi larut scFv dari *E.Coli*.

CHAPTER 1

INTRODUCTION

1.1 Epidemiology and Origin of HIV

Acquired Immunodeficiency Syndrome (AIDS) is one of the most serious diseases worldwide (Leeper and Reddi, 2010), it is caused by Human Immune deficiency Virus (HIV) (Sierra et al., 2005;Sarngadharan et al., 1984).

Estimating number of people living with HIV is 35.3 million in 2012 (UNAIDS,2013), wide separation of HIV with huge percent in middle and low-income countries lead to approximately 2 million infected patients dead every year. Of note, the highest percent of HIV in the world comes from sub-Sahara Africa with almost 67% of total world infections (UNAIDS, 2013).

Obviously, HIV-1 is transmitted from animals to humans especially from West and Central Africa (Hemelaar, 2012). HIV can be classified in two main types; firstly: HIV-1 and its related groups M, N, O and P and the second type: HIV-2 which has A-H group. HIV-1 are more prevalent and highly infective comparing to HIV-2 (Hemelaar, 2012).

1.2 Structure of HIV

Great efforts have been done to control AIDS epidemic through studying the biology, biochemistry and structural biology of HIV in order to understand the interaction between drugs and viral component (Turner and Summers, 1999).

HIV is a member of lentivirus genus which includes retrovirus species with complex genome. HIV's genome is encoded by RNA which converts to viral DNA when entering new host cell by viral reverse transcriptase (RT) (Ganser-Pornillos et al., 2008). HIV causes infection and destruction of CD4+ lymphocytes with in half-life of two days (Perelson et al., 1995).

Lentivirus spices, which composed of enveloped lipid bilayer derived from membrane of the host cell (Ganser-Pornillos et al., 2007). External surface glycoprotein (gp 120) are fixed to virus via interaction with transmembrane protein (gp41)(Ganser-Pornillos et al., 2007). Additionally the lipid bilayer may also contain some cellular membrane protein like actin, ubiquitin and major histocompatibility antigen (Arthur et al, 1992). The Matrix shell composed of around 2000 copies of matrix protein (P17) which lies in the inner surface of membrane of the virone, and the capsid core part ca. (P24) which composed of around 2000 copies of capsid protein and lies in the middle of the virus (Figure 1.2). Also, it includes nucleocapsid (P7) and three essential virally encoded enzyme Reverse Transcriptase (RT), Integrase(IN) and Protease(PT)(Turner and Summers,1999).

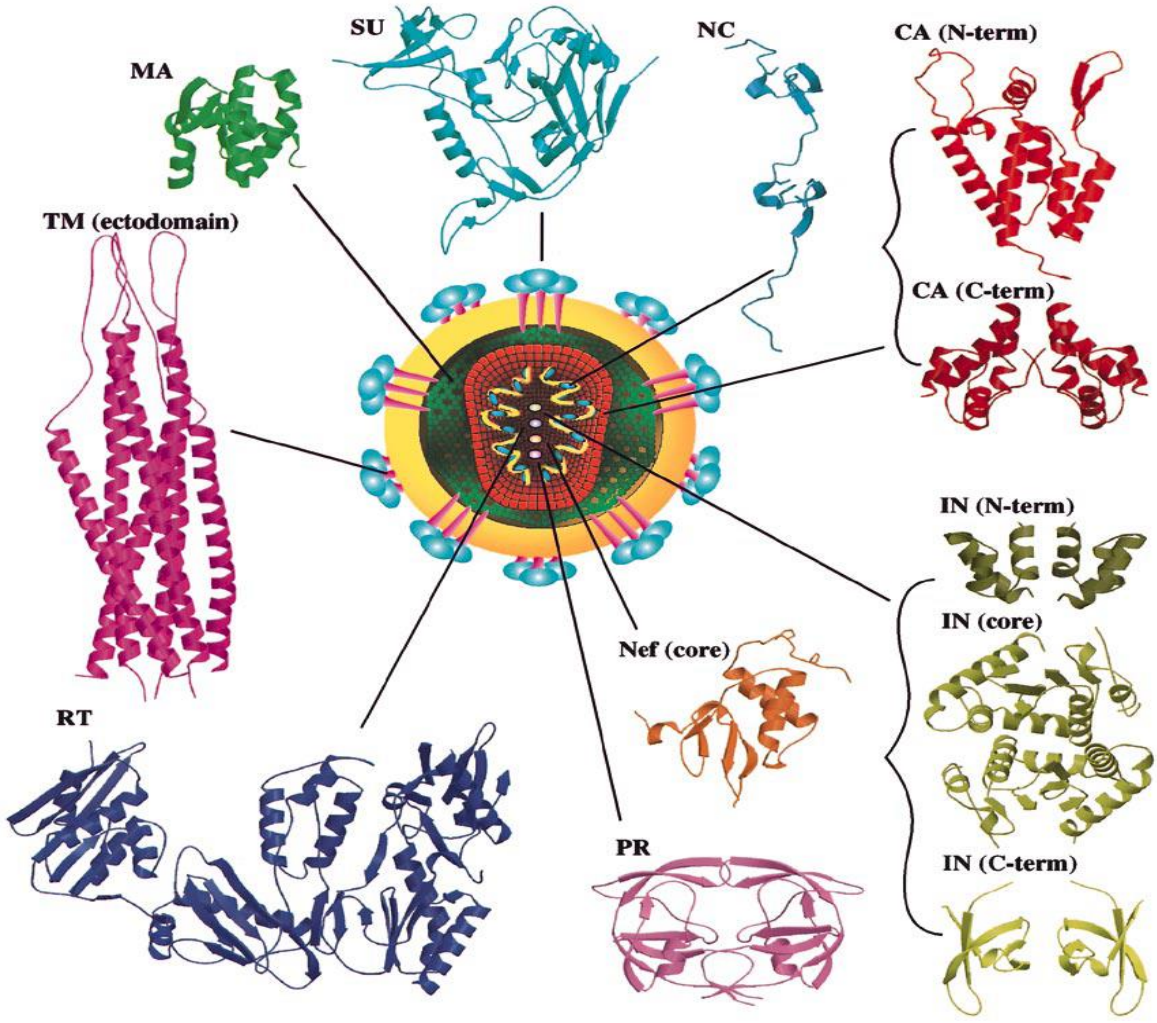

Figure 1.2. Structure of the mature HIV virion.

1.3 Mode of Transmission

HIV is transmitted mainly through three routes. Firstly, from mother to child which may be during pregnancy, delivery or breastfeeding (Vertical transmission). Secondly, through exposure to infected body or fluid, third route through sexual contact either homosexual or heterosexual (Markowitz, 2007). In addition ,there are links between HIV subtypes and transmission routes, a recent study which had been done in Uganda showed that the subtype D has lower rate of heterosexual transmission than subtype A. In addition, the subtype B infection can be constantly associated with intravenous drug abuse across the world (Hemelaar, 2013). The transmission route in America, Europe and Australia is mainly by heterosexual route which is associated with non B subtype and believed to be originated from Africa and Asia immigrants (G.Yebra et al, 2009). In India and Southern Africa, it is almost unique in subtype C to be caused from heterosexual transmission (C.Williamson, 1995). Of note, one of the transmission routes is mother-to-child transmission (MTCT), which had been shown in two large studies in Kenya and Uganda, the subtypes A and subtype D has higher rate of (MTCT) with no significant difference between them (Eshleman SH, et al.,2005; Murray MC et al., 2000).

1.4 Pathophysiology of HIV

Briefly, once the virus enters the body, it starts replication and killing T helper cells which plays an important role in adaptive immune response (Piatak,et al., 1993). An initial period of influenza like illness, with seroconversion then the patient develops the second stage which called asymptomatic infection or latent period that is characterized by falling of CD4 lymphocyte count to be less than 200 cells/ml of blood (Pantaleo G. et al., 1997). After a short period of time, the symptomatic stage of the disease begins, followed by AIDS caused by depletion of $CD4^+$ T cells which allows the opportunistic infection to attack the body without any defense leading to death at the end (Hel Z. et al, 2006).

1.5 Diagnosis of HIV

HIV/AIDS is diagnosed normally by laboratory testing as majority of patient develop seroconvert or specific antibody within three to twelve weeks of initial infection (CDC. 2012). Before antibody production it can be detected through HIV-RNA or P24 antigen, the positive result from both tests and for children especially below 18 months can be confirmed by PCR (CDC. 2012). For classification of HIV there are two main staging systems to use, either CDC classification system for HIV infection or WHO disease staging system for HIV infection and disease. The CDC system is more frequently used in developed countries. The center for disease, control and prevention (CDC) updated this system of classification in 2014, which rely on CD4 count and clinical symptoms for those patients older than 6 years (CDC. 2014).

- Stage 0: the time between a negative or indeterminate HIV test followed less than 180 days by a positive test.
- Stage 1: CD4 count ≥ 500 cells/µl and no AIDS defining conditions.
- Stage 2: CD4 count 200 to 500 cells/µl and no AIDS defining conditions.
- Stage 3: CD4 count ≤ 200 cells/µl or AIDS defining conditions.
- Unknown: if insufficient information is available to make any of the above classifications.

1.6 Prevention and Control

Firstly, form mothers to child (MTCT) or vertical transmission, there are programs to prevent this route which have shown great reduction in rate of transmission with approximately 92-99 %,(Horvath, T,et al.,2009). Those programs include using of antiretroviral during pregnancy and after delivery and switching breastfeeding to bottle feeding if it's convenient (Siegfried, N, et al., 2011).

Secondly, prevention of sexual contact by using condom, this has caused reduction of transmission rate by 80% (Crosby, R. et al., 2012). Also, both WHO and UNAIDS recommended male-circumcision as studies showed that it can reduce the rate of transmission of HIV by 38-66% (Siegfried, N.et al., 2009).

Finally, HIV patients whose CD4 count $\geq$ 350cells/μL can be treated with antiretroviral. This can protect 96% of their partner from infection (Chou R.et al., 2012). In addition, post exposure prophylaxis (PEP) can be controlled by a course of antiretroviral therapy within 48-72 hours after exposure to HIV positive patient or blood or genital secretion (Kuhar DT.et al., 2013).

1.7 Treatment of HIV

Indeed, up till now there are no treatment for HIV/AIDS which can eradicate the virus totally, although we have (ART) since 30 years (Chhatbar et al., 2011). Highly active antiretroviral therapy (HAART) works mainly through decreasing the virus progression and controlling its replication (Tab. 1.7). Also, it delays the progression of immunodeficiency which subsequently increases the life expectancy of the AIDS patients and decreases death rates (Broder, 2010; Chhatbar et al., 2011). Noteworthy, it's estimated that in developed countries and high-income ones like USA, (HIV-1) infected patients can live 14 years longer than expected if he/she continue on antiretroviral drug (Walensky et al., 2006; Vermund, 2006).

The basic antiretroviral drug and the first one ever was azidothymidine (AZT), which is nucleoside reverse transcriptase inhibitor (NRTIs) (Broder, 1990), after a period of time a new member of non-nucleoside reverse transcriptase inhibitor (NNRTIs) has joined (NRTIs) in order to attack HIV-1 RT site (Broder, 2010). Moreover, viral protease inhibitors were developed, besides a range of drugs which target different phases of viral replication process; like inhibition of early entry through fusion inhibition step using gp41 antagonist (Kilby et al., 1998), or even more earlier through blocking CCR5 co-receptor (Kuritzkes, 2009).

Although highly active antiretroviral therapy (HAART) is a good tool which convert (HIV-1) infection into chronic manageable disease, it is believed that (HAART) has a secondary side effect especially with aged patients under chronic treatment with (HAART) (Esté and Cihlar, 2010). Indeed, there are a lot of challenges regarding available regimen of

(HAART) as lack of efficacy, toxicity and incompatibility with other essential medications (Esté and Cihlar, 2010), so it is needed to find new tools to overcome these problems.

Brand name	Generic name(s)	Manufacturer name	Approval date	Time to approval
Nucleoside reverse transcriptase inhibitors (NRTIs)[a,b]				
Retrovir	Zidovudine, azidothymidine, AZT, ZDV	GlaxoSmithKline (original sponsor Burroughs-Wellcome)	19 March 1987	3.5 months
Videx	Didanosine, dideoxyinosine, ddI	Bristol Myers-Squibb	9 October 1991	6 months
Hivid	Zalcitabine, dideoxycytidine, ddC (no longer marked as of December 31, 2006)	Hoffmann-La Roche	19 June 1992	7.6 months
Zerit	Stavudine, d4T	Bristol Myers-Squibb	24 June 1994	5.9 months
Epivir	Lamivudine, 3TC	GlaxoSmithKline	17 November 1995	4.4 months
Combivir	Lamivudine and zidovudine	GlaxoSmithKline	27 September 1997	3.9 months
Ziagen	Abacavir sulfate, ABC	GlaxoSmithKline	17 December 1998	5.8 months
Videx EC	Enteric coated didanosine, ddI EC	Bristol Myers-Squibb	31 October 2000	9 months
Trizivir	Abacavir, zidovudine, and lamivudine	GlaxoSmithKline	14 November 2000	10.9 months
Viread	Tenofovir disoproxil fumarate, TDF	Gilead Sciences	26 October 2001	5.9 months
Emtriva	Emtricitabine, FTC	Gilead Sciences	02 July 2003	10 months
Epzicom	Abacavir and lamivudine	GlaxoSmithKline	02 August 2004	10 months
Truvada	Tenofovir disoproxil fumarate and emtricitabine	Gilead Sciences	02 August 2004	5 months
Nonnucleoside reverse transcriptase inhibitors (NNRTIs)[c]				
Viramune	Nevirapine, NVP	Boehringer Ingelheim	21 June 1996	3.9 months
Rescriptor	Delavirdine, DLV	Pfizer	4 April 1997	8.7 months
Sustiva	Efavirenz, EFV	Bristol Myers-Squibb	17 September 1998	3.2 months
Intelence	Etravirine	Tibotec Therapeutics	18 Jane 2008	6 months
Protease inhibitors (PIs)				
Invirase	Saquinavir mesylate, SQV	Hoffmann-La Roche	6 December 1995	3.2 months
Norvir	Ritonavir, RTV	Abbott Laboratories	1 March 1996	2.3 months
Crixivan	Indinavir, IDV,	Merck	13 March 1996	1.4 months
Viracept	Nelfinavir mesylate, NFV	Agouron Pharmaceuticals	14 March 1997	2.6 months
Fortovase	Saquinavir (no longer marketed)	Hoffmann-La Roche	7 November 1997	5.9 months
Agenerase	Amprenavir, APV	GlaxoSmithKline	15 April 1999	6 months
Kaletra	Lopinavir and ritonavir, LPV/RTV	Abbott Laboratories	15 September 2000	3.5 months
Reyataz	Atazanavir sulfate, ATV	Bristol-Myers Squibb	20 June 2003	6 months
Lexiva	Fosamprenavir calcium, FOS-APV	GlaxoSmithKline	20 October 2003	10 months
Aptivus	Tipranavir, TPV	Boehringer Ingelheim	22 June 2005	6 months
Prezista	Darunavir	Tibotec, Inc.	23 June 2006	6 months
Fusion inhibitors				
Fuzeon	Enfuvirtide, T-20	Hoffmann-La Roche and Trimeris	13 March 2003	6 months
Entry inhibitors—CCR5 co-receptor antagonists				
Selzentry	Maraviroc	Pfizer	06 August 2007	8 months
HIV integrase strand transfer inhibitors				
Isentress	Raltegravir	Merck & Co., Inc.	12 October 2007	6 months
Multi-class combination products				
Atripla	Efavirenz, emtricitabine and tenofovir disoproxil fumarate	Bristol-Myers Squibb and Gilead Sciences	12 July 2006	2.5 months

Tab. 1.7 Approved antiretroviral drugs.Adapted from: Drugs Used in the Treatment of HIV Infection, U.S. FDA. Drugs are listed in order of FDA approval within each class

1.8 Antibody and Antibody fragments (scFv)

Antibody is believed to protect the body against different types of infections and it is a naturally defense mechanism which has the ability to protect or even totally prevent the infection (Shukra et al., 2014). Antibodies were firstly discovered by Behring and Kitasato at 1983, where they found that infected animal can provide immunity against diphtheria, later on the door opened for the antibody production for Human therapy (Shukra et al., 2014). Eventually, immune sera from sheep, chickens, horses and humans can be collected and used as a treatment for some infectious diseases like, pneumococcal pneumonia, diphtheria and Tetanus (Casadevall A et al, 1994: Casadevall A, 1999).

Antibodies are found on the surface of B cells and extracellular fluids which can be used by the immune system to damage or kill bacteria or viruses (Sanz, L. et al. 2005). The basic unit of antibody is one immunoglobulin G (IgG) unit and compose of four chains, two identical light chains (L) and two identical heavy chains (H) with a molecular weight 150 KD (Nuñez-Prado et al. 2015).

Nowadays one of the most useful tools in research, diagnosis and therapy is Monoclonal antibodies (mAbs) (Sanz, L. et al. 2005). Obviously, by using molecular engineering technologies we can easily produce small and at the same time functional antibody. The single-chain variable fragments (scFv) contain both heavy chain (V_H) and light chain (V_L) which connected to each other by a flexible linker (Hagemeyer CE. 2009). The single-chain variable fragments (scFv) can be produced either in Eukaryote or prokaryote; however bacterial system is by far the best option regarding the good yield and low cost (Michael Steinitz, 2014).

Indeed, reducing condition of the *E-coli* bacterial cytoplasm make it difficult to form soluble scFv due to inability to form disulfide bond, however scFv can be produced either in periplasm or produced in cytoplasm as insoluble inclusion bodies that go further for a refolding process. In addition to that expression of scFv as a C-terminal fusion to maltose binding protein (MBP) can lead to high level production of stable and functional fusion protein.

1.9 Prokaryotic system

Recombinant Technology is one of the important tools in order to obtain a cheap, highly yielded and easily purified protein. The cheapest host that can be used is *Escherichia Coli* because it has different strains and produce good quantity of desired protein; however in many situations *E-Coli* can lead to production of insoluble and by default nonfunctional proteins (Vaks and Benhar, 2014). Soluble proteins can be obtained from recombinant *E-Coli*, by making some modification through engineering of target; like fusion tag technology.

In order to achieve soluble protein expression in *E.coli* cytoplasm, refolding of the inclusion bodies and purification process is quiet difficult and expensive comparing to purifying the highly expressed soluble protein (Vaks and Benhar, 2014). Therefore the best way is to produce a recombinant protein in a soluble form than to proceed with in vitro method. One of the good modification tools is reduction of temperature as aggregation reaction depends mainly on temperature, in addition the activity of some *E-Coli* chaperones have been increased on temperature around 30^0C, but the sudden decrease in temperature

can lead to inhibition of replication, transcriptional and finally translation. Also, low induction level can lead to higher amount of soluble protein (Vaks and Benhar, 2014).

1.10 Objective of this study

Main objective

To establish controlled intracellular processing of MBP-scFv in TEV*Eco* system for the production of functional scFv.

Specific objectives

1. To clone *anti-HIV1-CA scFv* gene with MBP as fusion partner in pSA expression vector.
2. To optimize expression of MBP-anti-HIV1-CA-scFv and its controlled intracellular processing in *TEVEco*
3. To characterize the purified anti-HIV1-CA-scFv.

CHAPTER II

MATERIALS AND METHODS

2.1 PCR amplification of both MBP-TEV using pRK793 plasmid and amplification of anti-HIV1-CA scFv using pHEN2 Ril-scFv46:

The PCR amplification was performed in order to amplify the MBP-TEV and anti-HIV1-CA scFv using specific primers. The PCR reactions were consisted of 5 X KAPA HiFi buffer, 2mM of Magnesium Chloride, mixture of dNTPs (0.3mM), and 10 ng of template DNA. 0.3 μM of forward and reverse primers.1X KAPA HiFi DNA polymerase. The PCR reaction was assembled on ice as following:

Component	Volume (μL)
5X KAPA HiFi buffer(1X)	10
dNTPs(0.2 mM)	1.5
Forward and reverse Primers(0.3 μM each)	3
Template DNA(10 ng)	2
KAPA HiFi DNA polymerase(1 U)	1
PCR water	32.5
Total	50

Tab. 2.1.1: PCR component for amplification of both MBP-TEV and amplification of anti-HIV1-CA scFv.

The contents of PCR tubes were vortexed followed by a brief spin using a microcentrifuge. The PCR tubes were then transformed to the thermocycler and cycling condition as follows: Initial denaturation at 95ºC for 3 minutes;10 cycles of (i) denaturation at 98 ºC for 20 seconds,(ii) annealing temperature of 65-55ºC for 15 seconds, (iii) extension at 72 ºC for 1 minute. This was followed by 25 cycles of denaturation at 98 ºC for 20 seconds at annealing temperature of 55 ºC for 15 seconds and final extension at 72 ºC for 5 minutes. The PCR amplified product underwent electrophoresis on 1% agarose gel and then visualized by placing into the gel documentation system (Chemiluminescence, VilberLourant) and the gel image was captured.

Primers used:

Primer	Template	Amplicon size(Kb)
MBP-NdeI-F1	pRK 793	1.2
TEV-SfiI-R1		
scFv-SfiI-F1	PHEN2-Ril-scFv-46	0.75
scFv-NotI-R1		

Tab.2.1.2 Primers used for amplification of both MBP-TEV and amplification of anti-HIV1-CA scFv.

1% Agarose Gel Electrophoresis:

1% agarose gel was prepared by adding 0.35 gram of agarose in 35 ml of 1 X Tris Acetic Acid EDTA (TAE) buffer. The solution was left for 5 minutes then heated at 50 level powers in a microwave oven for 1 minute to mix agarose until completely dissolved. The dissolved solution was cooled down until temperature equilibrated. Ethidium bromide was added into the agrose flask to a final concentration of 0.01mg/ml and the contents were mixed by gentle swirl. The gel casted by pouring 35 ml of agarose solution into the gel casting mold immediately followed by the insertion of sample loading comb. The gel was allowed to polymerize by setting it for 30 minutes at room temperature, then it was transferred to the electrophoresis unit. This was followed by adding 1 X TAE buffer followed by the removal of comb. Preparation of sample were done as follow: 1µl of DNA sample was mixed with 1µl of 6 X loading dye in addition to 4 µl of TAE buffer which was loaded in wells using micropipete. 1 µl of molecular weight markers (1Kb DNA Ladder of New England BioLab) + 1 µl 6 X loading dye + 4 µl of TAE buffer were also loaded simultaneously in the gel. The electrophoresis was carried out at 110 volts for 35 minutes, then DNA was visualized by placing into the gel documentation system (Chemiluminescence, VilberLourant) and the gel image was captured.

2.2-Clean-up for both anti-HIV-1 CA-scFv46 and MBP-TEV using SYBRE safe gel:

1% agarose gel was prepared by adding 0.35 gram of agarose in 35 ml of 1 X Tris Acetic Acid EDTA (TAE) buffer. The solution was left for 5 minutes then heated at 50 level powers in a microwave oven for 1 minute to mix agarose until completely dissolved. The dissolved solution was cooled down until the temperature equilibrated. 17.5µl of Sybre safe was added into the agrose to a final concentration and then contents were mixed by gentle swirl. The gel was casted by pouring 35 ml of agarose solution into the gel casting mold followed by the insertion of sample loading comb. The gel was then allowed to set for 30 minutes at 4ºC and covered. Then the gel was transferred to electrophoresis unit where 1 X TAE buffer was added, followed by the removal of comb. 50µl of DNA sample was mixed with 10µl of 6 X loading dye and carefully loaded into the wells using a micropipette. 1 µl of molecular weight markers (1kb DNA Ladder of New England BioLab) were also loaded simultaneously in the gel. The electrophoresis was carried out at 80 volts for 50 minutes. The DNA was visualized by placing into the gel documentation system (Chemiluminescence, VilberLourant) and the gel image was captured. Subsequently, the gel band of interest was cut using aseptic cutter under U.V light. 2ml tube was prepared and weighted before and after putting the gel, then it was spined for 13000xg for 1 minute, followed by dissolving the gel using block heater unit at 58ºC. The weight of the gel after dissolving was 383mg; subsequently the same amount of membrane binding solution was added, and then was put in block

heater for 2 minutes and vortexed, the last step was repeated 3 times. 1% agrose gel was put in 25ml TAE buffer.

2.3 Ligation process for insert MBP-TEV and anti-HIV1-CA scFv:

The MBP-TEV ration: to anti-HIV1-CA scFv was 1:1 for the most efficient ligation depends on the DNA concentration, the ligation mixture was set on ice. The ligation mixture was prepared as shown in this table**:**

Component	Volume(µl)
Restricted DNA(MBP-TEV) insert 100ng	1
Restricted DNA(anti-HIV1-CA scFv) insert 100ng	1
T4 DNA ligase buffer (10x)	1
T4 DNA ligase	1
dH2O	6
Total	10

Tab.2.3: Component of ligation Mix

The ligation mixture was prepared by vortexing then was spun in a microcentrifuge. Subsequently the ligation mixture was set in thermocycler and temperature was adjusted as follow:

Cycles = 99

10ºC/30 sec

11ºC/30 sec

12ºC/30 sec

13ºC/30 sec

14ºC/30 sec

15ºC/30 sec

16ºC/30 sec

17ºC/30 sec

18ºC/30 sec

End cycle

24ºC/ 2hr

Storage /pause/4ºC.

2.4 PCR amplification for insert MBP-TEV-anti-HIV1-CA scFv:

The PCR amplification was performed in order to amplify MBP-TEV- anti-HIV1-CA scFv using specific primers. The PCR reactions consisted of 5 X KAPA HiFi buffer, mixture of dNTPs (0.3 mM) and 10 ng of template DNA. 0.3 μM of forward and reverse primers. 1X KAPA HiFi DNA polymerase. The PCR reaction was assembled on ice as follow:

Component	Volume (μL)
5X KAPA HiFi buffer(1X)	10
dNTPs(0.3mM)	1.5
Forward and reverse Primers(0.3μM each)	3
Template(10ng)	1
1X KAPA HiFi DNA polymerase(1 U)	1
PCR water	33.5
Total	50

Tab.2.4.1: Component for PCR amplification for MBP-TEV-anti-HIV1-CA scFv

The contents of PCR tubes were vortexed followed by a brief spin using a microcentrifuge. The PCR tubes were then transformed to the thermocycler and cycling condition as follow:

Initial denaturation at 95ºC for 3 minutes;10 cycles of (i) denaturation at 98 ºC for 20 seconds,(ii) annealing temperature of 65-55ºC for 15 seconds, (iii) extension at 72 ºC for 1 minute. This was followed by 25 cycles of denaturation at 98 ºC for 20 seconds at annealing temperature of 55 ºC for 15 seconds and final extension at 72 ºC for 2 minutes. The PCR amplified product was electrophoretically on 0.7% agrose gel and then visualized.

Primers used:

Primer	Template	Amplicon size(Kb)
MBP-NdeI-F1	MBP-TEV-anti-HIV1-CA scFv	1.95 Kb
scFv-NotI-R1		

Tab.2.4.2: Primers used for PCR amplification for insert MBP-TEV-anti-HIV1-CA scFv

2. 5 PCR amplification of (pSA) vector using pMXB10-HP24-6His:

The PCR amplification was performed in order to amplify **(pSA) vector** using specific primers. The PCR reactions consisted of 5 X KAPA HiFi buffer, mixture of dNTPs (0.3 mM), and 10 ng of template DNA. 0.3 µM of forward and reverse primers.1X KAPA HiFi DNA polymerase. The PCR reaction was assembled on ice as follow:

Component	Volume (µL)
5X KAPA HiFi buffer (1X)	10
dNTPs(0.3mM)	1.5
Forward and reverse Primers(0.3µM each)	3
Template(10ng)	3
1X KAPA HiFi DNA polymerase (1 U)	1
PCR water	31.5
Total	50

Tab.2.5.1: Component for PCR amplification of (pSA) vector

The contents of PCR tubes were vortexed followed by a brief spin using a microcentrifuge. The PCR tubes were then transformed to the thermocycler and cycling condition as follow:

Initial denaturation at 95ºC for 3 minutes;10 cycles of (i) denaturation at 98 ºC for 20 seconds,(ii) annealing temperature of 65-55ºC for 15 seconds, (iii) extension at 72 ºC for 1 minute. This was followed by 25 cycles of denaturation at 98 ºC for 20 seconds at annealing temperature of 55 ºC for 15 seconds and final extension at 72 ºC for 2 minutes. The PCR amplified products were electrophoretically on 0.7% agrose gel and then it was visualized.

Primers used:

Primer	Template	Amplicon size(Kb)
6 His-Not1-F1	pMXB10-HP24-6His	5.3Kb
pMXB10-Nde1-R1		

Tab.2.5.2: Primers used for PCR amplification of (pSA) vector

2.6, 2.7 Purification and Gel clean-up for insert MBP-TEV-anti-HIV1-CA scFv46, and (pSA) vector, using Wizarad PCR Clean-up system:

Binding of DNA:

This was done using the same amount of membrane binding solution (50 µl) equal to the amount of PCR product (50 µl), SV minicolumn was inserted into collection tube, then the prepared PCR product was transferred to the minicolumn assembly and incubated for 1 minute, then it was centrifuged at 13000 xg for 1 minute, the precipitate was taken and

reload into Minicolumn assembly tube, then incubated for 1 minute and centrifuged at 13000 xg for 1 minute, the last step was repeated 2 times.

Washing:

700 µl membrane wash solution (ethanol) was added. Incubation was done for 1 minute then the mixture was centrifuged for another 1 minute at 13000 xg. then discard the flow through and reinsert Minicolumn into collection tube . Another 500 µl of Membrane Wash Solution was added and centrifuged at 13000 xg for 5 minutes; recentrifugation was done for 1 minute after discard the flowthrough.

Elution:

Minicolumn was carefully transferred to a clean 1.5 ml microcentrifuge tube, 10 µl of Nuclease free water was added to the Minicolumn, and then incubated for 1 minute and centrifuged at 13000 xg for 1 minute. Then last step was repeated 2 times. Finally the Minicolumn was discarded and DNA was stored at 4º C.

1% agarose gel was run after purification process, then proceed with 0.7% Sybre free gel in 50 ml (TAE), using 25µl of Sybre free, subsequently the specific band was cut and the clean-up process was continued as mentioned above.

2.8 Restriction digestion for both insert MBP-TEV-anti-HIV1-CA scFv and vector (pSA):

Restriction digestion for both insert and vector by preparing following component:

Component	Volume (µL)
Cut smart buffer(10X)	5
DNA(1µg)	21
Nde1 5U/µl	1
Not1 5U/µl	1
PCR water	22
Total	50

Tab.2.8.1: Component for restriction digestion for both insert and vector

The contents of PCR tubes were vortexed followed by a brief spin using a microcentrifuge. The PCR tubes were then transformed to the thermocycler and cycling condition was done at 37ºC for 8hr, and then storage was done at 4ºC.

Primer	Template
Not1	pMXB10-HP24-6His
Nde1	MBP-TEV-anti-CA-scFv46

Tab.2.8.2: Primers used for restriction digestion for both insert and vector