생명과학회지
Korean J. Life Science, 9(5), 556~563, 1999

Expression of Human Immunodeficiency Virus Type 1 Gag Protein in *Escherichia coli*

Weon Sang Choi†

Department of Biochemistry, College of Natural Science, Dongguk University, Kyongju 780-714, Korea

Abstract

Presence of antibody to the capsid protein p24 is the main diagnostic criterion, since this reflects reliable antibody response to HIV infection. However, it takes about 6-8 weeks for antibody production after infection and people who are infected but antibodies are not produced yet are classified as seronegative. Therefore, there is a strong need for an improved diagnostic method for better health security. As a first step for developing such an improved diagnostic system, gag protein of human immunodeficiency virus type 1 was expressed in *E. coli* DH5α. The *gag* fragment of HIV-1 (including a portion of p17 and whole p24) was amplified by polymerase chain reaction (PCR) and *Bam*H I/*Eco*R I sites were created during PCR. The amplified DNA fragment was cleaved with *Bam*H I/*Eco*R I and was subcloned into the GEX-2T vector which had been digested with *Bam*HI/*Eco*RI, resulting gene fusion with *gst* gene of pGEX-2T. The recombinant DNA was transferred into *E. coli* DH5α. The transformed bacteria were grown at 37°C for 3h and protein expression was induced with 0.1mM IPTG at 25°C for 3h. Recombinant gag protein or GST-gag fusion protein was purified with glutathione-sepharose 4B bead and migrated as a single band when analyzed by 10% polyacrylamide gel. These proteins were confirmed by immunoblotting with anti-GST goat sera or Korean AIDS patients sera. The results of this study establish the expression and single step pulification of HIV-1 gag protein which can specifically bind with Korean AIDS patients sera.

Key words – Capsid, Gag protein, Polymerase chain reaction, Immunoblotting

Introduction

During the past 20 years, acquired immunodeficiency syndrome (AIDS) has became a big threat to mankind. AIDS is belived to be caused by human immunodeficiency virus (HIV). The World Health Organization (WHO) estimated that by late 1993 HIV has already infected about 10 million people worldwide[6]. The number of infected people in Korea continue to increase every year and 570 cases of HIV infection were reported by mid 1996[7].

HIV is taxonomically classified as a membrane of the family *Retroviridae*. The family *Retroviridae* consists of several subfamilial genus. HIV is included in the subfamilial genus *Lentivirus*[2]. HIV genome has 2 long terminal repeats (LTR), 3 major genes (*gag, pol,* and *env*) and additional reguratory genes (*rev, tat, nef, vif, vpr, vpu, vpx*). Biochemical and immunochemical analysis has demonstrated that the nucleocapsid within each mature virion is composed of two molecules of the viral single-stranded RNA genome encapsulated by proteins proteolytically processed from the gag precursor polypeptide. Cleavage products of the *gag* gene polyprotein Pr55 gag by the viral protease include matrix protein MA (p17)

† Corresponding author

which is located between the nucleocapsid and viral envelope, capsid protein CA (p24) which forms the capsid shell, and nucleocapsid protein NC (p9) which binds to the viral RNA genome[5,10,13].

Most of HIV-infected people develop symptoms of AIDS between 8 and 10 years after infection, but it is not possible to predict how quickly any given infected individual will develop AIDS symptoms[4,8,11]. Presence of antibody to the capsid protein p24 is the main diagnostic criterion, since this reflects the infection of HIV. If the antibody to p24 is detected in an enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA), the person is classified as HIV sero-positive[6]. Although so far the detection of antibody to p24 has been successful for diagnosis, there is a serious problem for screening infected people because the infected but antibodies are not produced yet, which usually takes 6-8 weeks after infection, are classified as sero-negative[3], meaning the test needs to be improved for better health security.

In this report, as a first step for develoing improved diagnostic system, gag p24 gene was expressed in *E. coli*. The *gag* of HIV-1 was amplified by polymerase chain reaction (PCR) and was subcloned into the GEX-2T vector (Pharmacia). The recombinant DNA was transformed into *E. coli*. Protein expression was induced with IPTG and GST-gag fusion protein or gag protein was purified and was tested whether Korean AIDS patient sera can bind with.

Materials and Methods

Materials

Restriction enzymes and DNA modification enzymes were purchased from Promega. The Taq DNA polymerase and reagents for the PCR were obtained from Takara. Sequanase, GST purification modules, GST detection module, ECL western blotting analysis system, and Hyperfilm ECL were purchased from Amersham Pharmacia biotech. Synthetic DNA oligomers were prepared from GenoTech (Taejon, Korea). Pooled sera from Korean AIDS patients were a gift from Jinseu Park (Hallym university). A rabbit polyclonal antibody specific for human or goat IgG was obtained from Sigma Co. All chemicals unless otherwise noted were purchased from Sigma Chemical Co.

Methods

Construction of HIV-1 Gag protein expression plasmid

All DNA manipulations were performed according to standard procedures[12]. Restriction enzymes and DNA modification enzymes were used according to the manufacturer's instructions. The plasmid used to express the authentic HIV-1 gag protein or as a fusion with glutathione S-transferase (GST) was constructed as follows. A DNA fragment of the HIV-1 *gag*, corresponding to nucleotides 921 to 1891(a portion of p17 plus whole p24) was amplified by PCR (Perkin-Elmer 2400, Foster, CA) with the synthetic DNA primers DG28 and DG29 (Table 1). The DNA primers used for PCR amplification were designed to create *Bam*H I and *Eco*R I for subcloning into the pGEX-2T vector. For the PCR reactions, the HIV-1 plasmid pNL4-3 was used as the template. The thermocycler conditions were set at 94°C (1 min), 42°C (1 min) and 72°C (1 min) for a total of 30 amplification cycles. One-tenth of the product was analyzed on 0.8% agarose gels to ensure the amplification. The amplified DNA fragment was cleaved with *Bam*H I/*Eco*R I, and was ligated into the pGEX-2T plasmid which had been previously digested with *Bam*H I/*Eco*R I. Following transformation into *E. coli* DH5α, the colonies were screened for the presence of the HIV-1 *gag* DNA fragment by restriction digestion. After sequencing, the final clone was designated pGEX-2T-gag.

DNA sequencing

For sequencing, DNA was prepared by using Wizard Minipreps DNA purification system (Promega) and sequenced by using Sequenase version 2.0 sequencing

Table 1. List of oligonucleotides used in this work

Oligonucleotides	Polarity	Sequence (5′ → 3′)
DG 28	sense	AGGGAGCTAGAA**GGATCC**GCAGTTAATCCT
DG 29	antisense	AGCTGGATTTGT**GAATTC**GCTCATTGCTTC
DG 8	sense	GGGCTGGCAAGCCACGTTTGGTG
DG 9	antisense	CCGGGAGCTGCATGTGTCAGAGG

kit (Amershampharmacia biotech) with primers DG8 and DG9.

Expression of GST and GST-Gag fusion proteins

GST and GST-gag fusion proteins were expressed in *E. coli* DH5α. Bacterial cultures were grown for 3 h at 37°C in LB medium and protein expression was induced with 0.1mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 25°C. The bacterial cells were then collected by centrifugation and lysed by freezing / thawing in 5 volumes of PBS buffer and sonicated. The bacterial extract was then clarified by centrifugation. The supernatant was then incubated with glutathione resin (Pharmacia) for overnight at room temperature. The glutathione beads were washed sequentially with PBS buffer. Bound GST or GST-gag fusion protein was eluted with 5 volumes of 10mM reduced glutathione. The eluted proteins were used directly or treated with thrombin.

SDS-polyacrylamide gel electrophoresis and immunoblotting

The concentration of the protein was determined by Follin-Lowry method[9]. The proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by coomassie brilliant blue staining. For immunoblotting, proteins from SDS-PAGE were transferred to nitrocellulose membranes by using the semidry transfer cell (Trans-Blot SD, BioRad). The membranes were blocked overnight with blocking buffer (80mM sodium phosphate dibasic, 20mM sodium phosphate monobasic, 100 mM NaCl, 0.3% Tween 20, 5% low fat dried milk) and incubated for 4 to 6 h at room temperature or overnight with goat anti-GST antibodies (1 : 1000) or heat inactivated pooled Korean AIDS patients sera (1: 6000). This was followed by three 10 min washes with PBST (80 mM sodium phosphate dibasic, 20 mM sodium phosphate monobasic, 100 mM NaCl, 0.3% Tween 20). Secondary antibody (rabbit anti-goat or rabbit anti-human: Sigma Co.) was added (1 : 1000), and the membranes were incubated for additional 4h and then sequentially peroxidase-conjugated donkey anti-rabbit antibodies (ECL; Amershampharmacia biotech) were treated for 4 to 6 h at room temperature. After three 15 min washes with PBST, membranes were reacted with chemiluminescence reagent (Amershampharmacia biotech) and exposed to hyperfilm ECL.

Results

The pNL4-3 is a recombinant proviral clone that contains DNA from HIV-1 isolates NY5 and LAV[1]. The *gag* of pNL4-3 is derived from NY5. Therefore, the amplified *gag* used in this study is NY5 origin. pGEX-2T vector has unique *Bam*H I and *Eco*R I restriction endonuclease cleavage site to permit the expression of sequences introduced into the vector. A DNA fragment of the HIV-1 *gag*, corresponding to nucleotides 921 to 1891(a portion of p17 plus whole p24) was amplified by PCR (Fig. 2A). *Bam*H I and *Eco*R I site for subcloning into the pGEX-2T vector were created during PCR amplification. The amplified DNA fragment was cleaved with *Bam*H I/*Eco*R I, and was ligated into the pGEX-2T vector which had been previously digested with *Bam*H I/*Eco*R I. The resulting plasmid pGEX-2T-gag contains a

gag as a fusion with glutathione S-transferase (GST) gene and a β-lactamase gene for selective growth in ampicillin-containing media (Fig. 1, Fig. 2B). Because the fusion gene is under the control of the *tac* promoter, the lacose analog isopropyl β-D-thiogalactoside (IPTG) was used to induce the fusion protein. The plasmid pGEX-2T-gag was sequenced to confirm that the inserted sequences have correct bases and that the junction regions between *gst* and *gag* conserve translational reading frame (Fig. 3).

The expression of HIV-1 gag protein from GEX-2T-gag was next determined (Fig. 4A, 4B). The GST-gag fusion proteins were easily detected from sonicated lysate by staining with coomassie brilliant blue (Fig. 4A lane 3). In the preliminary experiments, GST-gag fusion protein induction was not much dependent on the IPTG concentration (tested from 0.025 mM to 3 mM) and 0.1 mM was chosen for next experiments (data not shown). The glutathione-Sepharose 4B beads were added to the supernatant of the sonicated bacterial lysate and bound CST or CST-gag proteins were eluted with reduced glutathione. The eluted proteins were electrophoresed directly or after treated with thrombin. The eluted GST-gag fusion protein was almost pure and migrated as single band. (Fig. 4B lane 5). Two bands were apparent from GST-gag after thrombin digest (Fig. 4B lane 6). However, the molecular weight of expressed gag protein (approximately 27 kDa) was smaller than calculated

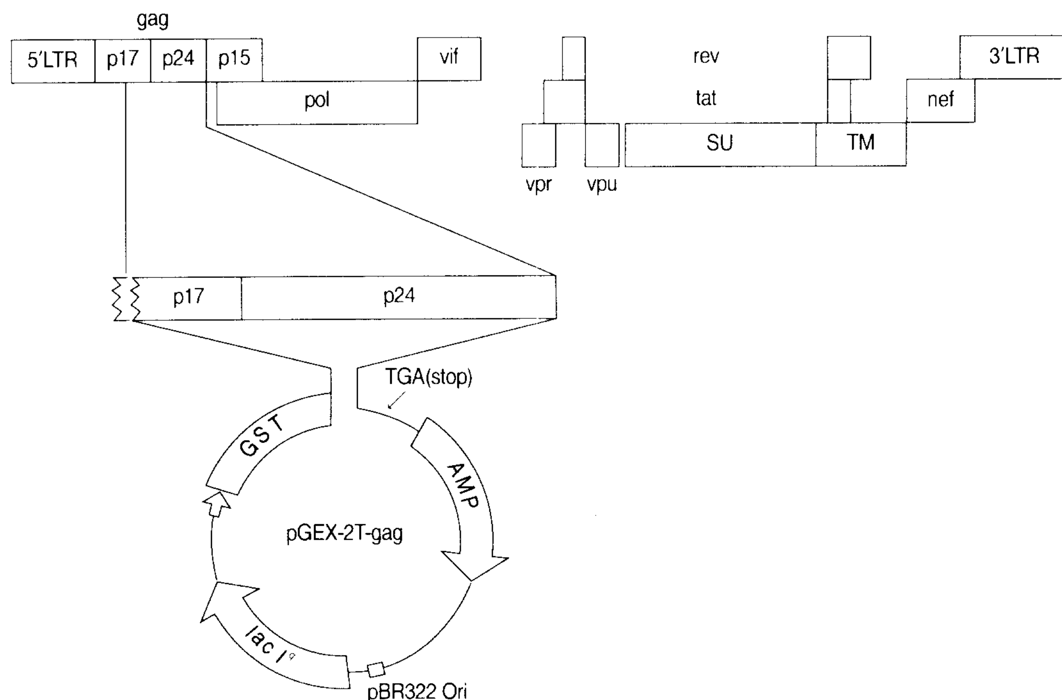

Fig. 1. Construction of HIV-1 gag protein expression vector pGEX-2T-gag.
A segment of HIV-1 *gag* which includes a portion of p17 and whole p24 was amplified by PCR and cleaved with *Bam*H I/*Eco*R I. It was ligated into the pGEX-2T vector which had been digested with *Bam*H I/*Eco*R I so that the translational reading frame was conserved between the *gst* and *gag*. Protein expression is under the control of *tac* promoter, which is induced by IPTG.

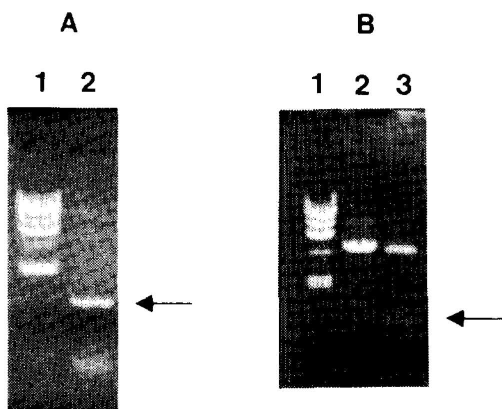

Fig. 2. **A.** Agarose gel electrophoresis of HIV-1 gag DNA amplified by PCR. Arrow indicates the PCR product, whose molecular size is approximately 900 bases. The segment of DNA was amplifieded by using primer DG28 and DG29 and *Bam*H I/ *Eco*R I sites were created during amplification. Lane 1, size marker (λ DNA digested by *Hind* III); lane 2, HIV-1 gag. **B.** Restriction enzyme digestion of pGEX-2T-gag by *Bam*H I/*Eco*R I. Inserted DNA fragment is marked. Lane 1, size marker (λ DNA digested by *Hind* III); lane 2, pGEX-2T; lane 3, pGEX-2T-gag.

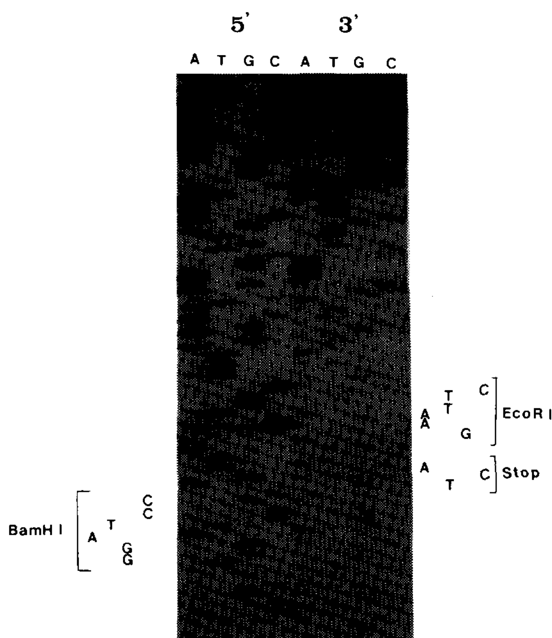

Fig. 3. Nucleotide sequences of pGEX-2T-gag, which shows junction regions between *gst* and *gag* gene. *Bam*H I and *Eco*R I sites created during amplification are marked.

(approximately 33 kDa) based upon the cDNA sequence with the notion that proteolytic cleavage of the expressed protein occurred at the recognition sequences for thrombin protease.

To confirm whether the induced protein from pGEX-2T-gag transformed bacteria is really GST-gag fusion protein, immunoblot analysis was performed (Fig. 5A, 5B). Goat sera against GST recognized the GST or GST-gag fusion protein (Fig. 5A, lanes 4,5,6). The HIV-1-related proteins, representing an GST-HIV-1 gag fusion protein or gag protein were specifically recognized by Korean AIDS patients sera (Fig. 5B, lanes 3,4). No immunoreactive proteins were detected from DH5α or GEX-2T transformed DH5α (Fig. 5B, lanes 3,4).

Discussion

In this work, the expression and purification of HIV-1 gag protein in *E. coli* was performed. The HIV-1 gag is relatively less polymorphic and the protein itself is stable compared with other HIV-1 proteins. At present the presence of antibody to the capsid protein gag p24 is main diagnostic criterion. During the first few weeks after infection, gag antigens are present in serum but antibodies are not produced yet and people infected by HIV within 6-8 weeks can be classified falsely as seronegative when using the current test kit. Therefore, current test kit has a severe problem and there is a strong need for an improved diagnostic method for better health security. This work is a first step for solving this problem and long term goal is to develope a diagnostic system to detect minute amount of antigen in the human blood before antibodies are produced. Segments of HIV-1 proviral DNA containing the *gag* was inserted into the pGEX-2T vector so that the translational reading frame was conserved between the

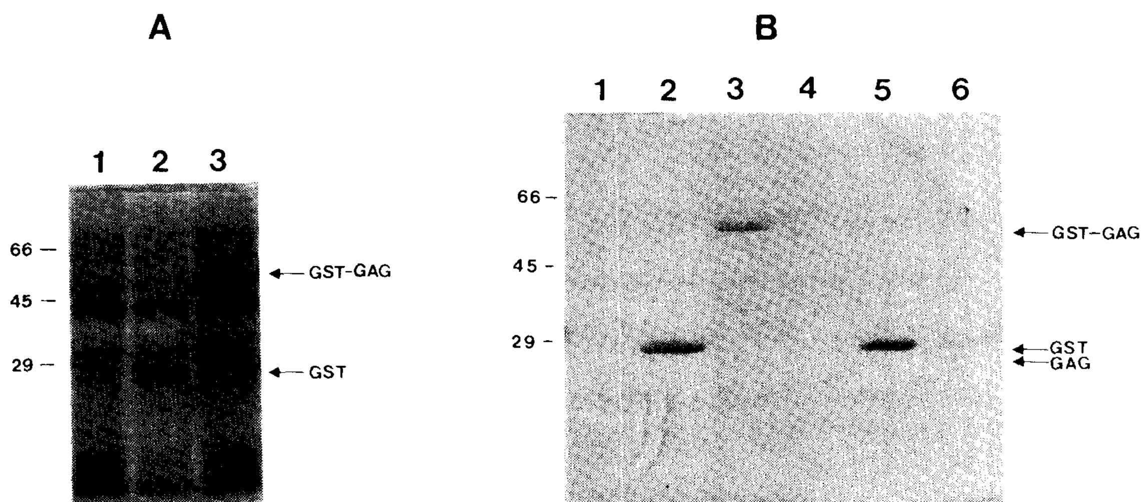

Fig. 4. **A**. Expression of HIV-1 gag or related proteins in *E. coli* DH5α harvoring the *gst-gag* fusion gene. Proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie brilliant blue. **A.** SDS-PAGE of whole cell lysate. Lane 1, host bacteria DH5α; lane 2, pGEX-2T transformed bacteria; lane 3, pGEX-2T-gag transformed bacteria. **B.** SDS-PAGE of purified proteins. Lanes 1, 2, and 3, proteins were purified with glutathione-Sepharose 4B bead. Lanes 4, 5, and 6, proteins purified with glutathione-Sepharose 4B bead and then treated with thrombin. Lane 1, proteins from host bacteria DH5α; lane 2, proteins from pGEX-2T transformed bacteria; lane 3, proteins from pGEX-2T-gag transformed bacteria; lane 4, proteins from host bacteria DH5α; lane 5, proteins from pGEX-2T transformed bacteria; lane 6, proteins from pGEX-2T-gag transformed bacteria.

gst and HIV-1 *gag*. However, the plasmid DNA when transformed into *E. coli* DH5α expressed the smaller HIV-1 gag protein than calculated based upon the cDNA sequence (Fig 4B, lane 6; Fig. 5B lane 4). In order to verify the reason, the whole cloned *gag* region of GEX-2T-gag was sequenced and found no stop codon within the open reading frame (data not shown). At present it is not clear why there exists discrepancy in molecular weight between what was expected and what was migrated in the gel electrophoresis. One possible explanation might be a cleavage by host protease. For the Gag protein expression, the induction temperature was critical. When the induction was performed at 37°C, most of the fusion protein was found in the pellet as a insoluble inclusion body. However, in case induced at 25°C, most of the fusion protein was recovered in the supernatant of the sonacated lysate (data not shown).

The result of this study establish the expression and single step purification of HIV-1 gag protein which can specifically react with Korean AIDS patients sera. The HIV-1 gag protein from bacteria transformed with pGEX-2T-gag is the result of the translation of the GST-gag fusion protein and subsequent cleavage by the thrombin protease. From the scale-up experiment of 250 ml culture, about 0.5 mg of almost pure HIV-1 gag protein was recovered (data not shown). Further works are going on to develope rabbit antibody using the purified gag protein and ELISA plates which can detect the viral gag antigen from the blood of HIV-1 infected people before antibodies are raised.

Acknowledgements

I would like to appreciate Dr. Kyong-Tai Kim

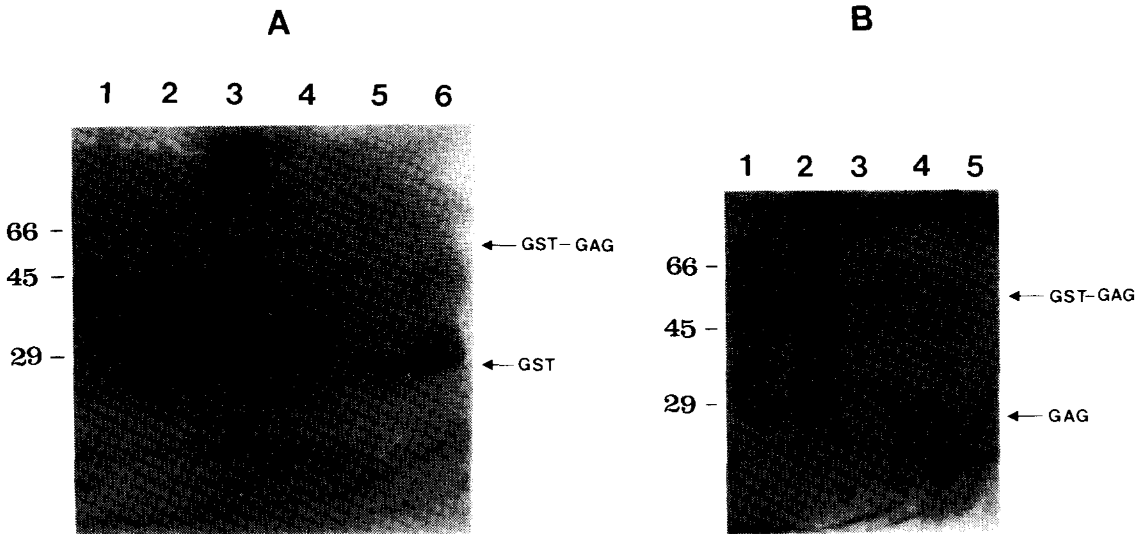

Fig. 5. Immunoblot analysis of the HIV-1 gag or related proteins.

Proteins were purified with glutathione-Sepharose 4B bead, fractionated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane. **A.** The membrane was reacted sequentially with anti-GST goat serum, anti-goat rabbit IgG and peroxidase labeled anti-rabbit donkey IgG. The membrane was reacted with ECL detection reagents and exposed to Hyperfilm ECL. Lane 1, whole cell lysate of host bacteria DH5α. Lane 2, whole lysate of pGEX-2T transformed bacteria. Lane 3, whole lysate of pGEX-2T-gag transformed bacteria. Lane 4, proteins from pGEX-2T-gag transformed bacteria which was purified with glutathione-Sepharose 4B bead. Lane 5, protein from pGEX-2T-gag transformed bacteria which was purified with glutathione-Sepharose 4B bead and then cleaved with thrombin. Lane 6, protein from pGEX-2T transformed bacteria which was purified with glutathione-Sepharose 4B bead and then cleaved with thrombin. **B.** The membrane was reacted sequentially with Korean AIDS patients serum, anti-human rabbit IgG and peroxidase-labeled anti-rabbit donkey IgG. The membrane was reacted with ECL detection reagents and exposed to Hyperfilm ECL. Lane 1, whole cell lysate of pGEX-2T transformed bacteria; lane 2, whole cell lysate of pGEX-2T-gag transformed bacteria; lane 3, protein from pGEX-2T-gag transformed bacteria, which was purified with glutathione-Sepharose 4B bead; lane 4, protein from pGEX-2T-gag transformed bacteria, which was purified with glutathione-Sepharose 4B bead and then cleaved with thrombin; lane 5, protein from pGEX-2T transformed bacteria, which was purified with glutathione-Sepharose 4B bead and then cleaved with thrombin, showing no protein band.

(Postech) and Dr. Jinseu Park (Hallym univ.) for providing some reagents. I also appreciate Jung-Un Yoon for technical assitance. This work was supported by the Journal publication/research fund from Dongguk University.

References

1. Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson and M. A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Uirol.*, **59**, 284-291.
2. Coffin, J. M. 1996. *Retroviridae*: The viruses and their replication, chapter 58, In B. Fields(ed.) *Virology*, Lippincott-Raven Publisher Philadelphia-New York.
3. Cooper, D. A., A. A. Imrie and R. Penny. 1987. Antibody response to human immunodeficiency virus after primary infection. *J. Infect. Dis.*, **155**, 1113-1118.
4. Curran, J. W., H. W. Jaffe, A. M. Hardy, W. M. Morgan, R. M. Selik and T. J. Dondero. 1988. Epidemiology of HIV infection and AIDS in the United States. *Science*, **239**, 610-616.
5. Henderson, L. E., M. A. Bowers, R. C. Sowder, S. A. Serabyn, D. G. Johnson, J. W. Bess, L. O. Arthur, D. K. Bryant and C. Fenselau. 1992. Gag proteins of the highly replicative MN strain of human immunode

ficiency virus type 1: posttranslational modifications, proteolytic processing, and complete amino acid sequences. *J. Virol.*, **66**, 1856-1865.
6. Hirsch, M. S. and J. Curran. 1996. Human Immunodeficiency Viruses, chapter 61, In B. Fields(ed.) *Virology*, Lippincott-Raven Publisher Philadelphia-New York..
7. Kim, Y. B., Y. K. Cho, H. J. Lee, K. H. Chung, J. W. Kim, Y. K. Kim and J. M. Yang. 1996. Sequence and phylogenetic analysis of V3 region of human immunodeficiency virus type 1 strains isolated from Korean patients. *J. Kor. Soc. Virol.*, **26**, 251-258.
8. Levy, J. 1993. HIV pathogenesis and long term survival. *AIDS*, **7**, 1401-1410.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with Follin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.
10. Luciw, P. A. 1996. Human Immunodeficiency Viruses and their replication, chapter 60, In B. Fields(ed.) *Virology*, Lippincott-Raven Publisher philadelphia-New York.
11. Ragni, M. V. and L. A. Kingsley. 1990. Cumulative risk for AIDS and other HIV outcomes in a cohort of hemophiliacs in western Pennsylvania. *J. AIDS*, **3**, 708-713.
12. Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. *Molecular cloning. A laboratory manual.* 2nd Ed. Cold Spring Harbor Laboratory Press.
13. Sanchez-Pescador, R., M. D. Power, P. J. Barr, K. S. Steimer, M. M. Stempien, S. L. Brown-Shimer, W. W. Gee, A. Renard, A. Randolph, J. A. Levy and P. A. Luciw. 1985. Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). *Science*, **227**, 484-492.

초록 : 대장균을 이용한 후천성 면역결핍바이러스 Gag단백질의 발현

최원상
(동국대학교 자연과학대학 생화학과)

혈액내에 HIV의 capsid 단백질 p24에 대한 항체존재 여부가 HIV감염여부를 반영해 주므로 일반적으로 이 원리를 진단시약제조시 이용하고 있다. 그러나 감염후 항체를 생성하는데는 적어도 6-8주가 소요되므로 비록 감염이 되었다 하더라도 아직 항체가 생성되지 않은 사람은 sero-negative로 잘못 분류된다. 따라서 국민보건의 향상을 위해서는 보다 나은 진단시약의 개발이 요구된다. 이 같은 진단시약의 개발을 위한 첫단계로 HIV-1의 gag단백질을 대량 생산하고자 대장균에서의 발현을 시도하였다. 이를 위해 HIV-1 pNL4-3의 gag유전자를 PCR을 이용하여 증폭한 후 이를 대장균 발현백터에 GST유전자와 융합되게 subcloning한후 이를 이용하여 대장균을 transformation하였다. 이 대장균을 37°C에서 3시간 키운후 다시 25°C에서 IPTG를 이용하여 3시간 동안 induction하였다. 생성된 단백질은 glutathione-sepharose 4B bead를 이용하여 정제하였고 10% SDS-PAGE로 확인 결과 단일 band로 이동하였다. 이를 immunoblot으로 확인 결과 한국인 AIDS환자의 혈장과 특이하게 결합함을 알 수 있었다. 이 결과로 HIV-1 gag 단백질이 대장균에서 다량 발현될 수 있고 한번의 정제로 HIV-1과 특이하게 결합할 수 있는 높은 순도의 단백질을 얻을 수 있음을 알수 있었다.