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NOVEL METHOD FOR THE DETECTION OF HIV USING RP24 RECOMBINANT PROTEIN FROM *E. COLI* BL-21

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ABSTRACT

A novel method for the purification of recombinant protein 24 (rp24) of human immunodeficiency virus type 1 (HIV- 1) from *Escherichia coli* is described. The protein was over-expressed in *E. coli* (BL21 lDE3) cells to the levels of ~ 30 % total cell protein in the soluble fraction. The recombinant protein was extracted and purified to near homogeneity by ion-exchange steps followed by ultrafiltration. The recombinant protein in combination with synthetic peptides from immuno-dominant stretches derived from the 'envelope' proteins of HIV-1 and HIV-2 was used for the development of an ELISA kit, for detection of antibodies against the virus in human serum and/or plasma.

KEYWORDS

ELISA, rp24, synthetic peptides, ultrafiltration

INTRODUCTION

Escherichia coli is the most widely used prokaryotic system for the synthesis of heterologous protein due to its well-documented genetics and physiology[1]. Achieving high expression levels of target protein is one of the main objectives in the cultivation of recombinant cells. This objective may be accomplished through two complementary approaches, namely, genetic engineering and optimal induction. Human immunodeficiency virus (HIV) is the etiological agent for acquired immunodeficiency syndrome (AIDS) which has already been separated into distinct subtypes[2]. Of the two known HIV types, HIV type 1 (HIV-1) is the predominant virus worldwide. Currently, HIV infection and AIDS has become one of the major public health problems in the world. Prevention of infection from HIV is perhaps the best way to stop its spread among populations as no commercial vaccine is available as yet and the multidrug-resistant strains of the virus are making the treatment increasingly difficult [3].

Emergences of multidrug-resistant viral strain, non-availability of an effective vaccine and inappropriate social behaviors of populations have caused the HIV pandemic across astounding proportions. There are around 3.6 million HIV infected persons globally and more than 15,000 new infections are being added daily [4]. Early diagnosis of the infection is thus of utmost importance for counseling, prevention of spread and ensuring safety of blood obtained from donors. Hence, screening of blood donors and high risk population by

highly sensitive and specific methods is essential. Although various methods are available for diagnosis of HIV infection, serologic determination of circulating anti-HIV antibodies using various ELISA methods is still preferred as the primary screening technique[5].

The basic genes of the virus are consisted of *gag*, *pol* and *env*, which encode matrix, polymerase/reverse transcriptase and envelope proteins, respectively [2]. The *env* glycoprotein of HIV-1, gp160 is prototypically processed by a host protease to generate the surface (SU) and trans-membrane (TM) subunits of the mature *env* glycoprotein complex that are designated as gp120 and gp41[6]. The trans-membrane protein gp41 of HIV-1 is the most important antigen for sensitive and specific detection of anti-HIV antibody[7]. HIV-1 *gag* protein precursor for p55 is processed to p17 (matrix protein), p24 (core protein) and p15 [8]. The non-glycosylated 24 kDa protein, p24 forms the core of the virus and encapsulates the viral genetic code [9]. Among *gag* protein precursors, the p24 antigen and its antibody (anti-p24) have been generally applied for determination of HIV infection by Western blot and other serological assays as ELISA[10- 12].

In order to create HIV detecting serological assay systems such as ELISA and Western blot, the gp41 and *gag* or its fragments, p15, p17 and p24 have been produced in a wide variety of expression systems, including *E. coli* [8,13-15], *Saccharomyces cerevisiae*[16], recombinant baculovirus[17], adenovirus[18], Chinese hamster ovary cells[19], as well as natural production systems[20].

We describe here cloning, over expression in *Escherichia coli*, and a simple, novel method for purification of recombinant p24 (rp24) protein of HIV-1. The purified rp24 was used in the development of an ELISA kit, for detection of antibodies to HIV-1 and HIV-2 in patient serum.

MATERIALS AND METHODS

1. Transformation of the strain

E. coli strain BL21 DE3 cells were transformed with the recombinant plasmid DNA containing pET 23b vector (Ampicillin [Sigma Aldrich] resistant). A loopful of colonies from the LBamp plate was inoculated in 5 ml LB medium containing ampicillin. The culture was grown at 37 ^{0}C and at 200 rpm till the OD600 nm reached 1.0.

2. Induction and harvesting of the culture

Protein expression was induced with 1 mM iso-propyl- α- D thiogalactoside (IPTG from USB) for 90 min at 37 ^{0}C and 200 rpm. The cells were harvested and lysed by the addition of Lysozyme (USB). The lysate was clarified by centrifugation at 2000 x *g*. The clarified lysate was subjected to centrifugation at 1000 x *g* and the supernatant and pellet fractions were used for studying the localization of the recombinant protein.

3. Purification of protein

For protein purification, transformed *E. coli* cells were grown in 1 l LB broth at 37 ^{0}C till OD600 nm reached ~ 1.0.

4. Expression of rp24 protein

The expression of rp24 was induced with 1 mM IPTG for 3 h. Cells were harvested, suspended in 20 mM Tris (USB) pH 7.4 containing 1 mM EDTA (MERK), pH 8.0, subjected to lysis by addition of Lysozyme and centrifuged at 2000 x *g*. The cytosolic fraction containing the protein was prepared by centrifugation of the clarified lysate at 10000 x *g*.

5. Purification of the lysate containing protein by HPLC and ultrafiltration

The supernatant was loaded on an anion exchange column (20 ml, Q-Sepharose, Amersham-Pharmacia, USA) at a flow rate of 3 ml/min. The column was washed with 5 column volumes (CV) of buffer A (20 mM Tris-HCl, pH 7.4) and the bound protein was eluted with a linear salt gradient (0–50%, 5 CV) of buffer B (buffer A containing 0.5 M NaCl). The Q-

Sepharose column fractions were analyzed by 12.5 % SDS-PAGE for the presence of rp24. Fractions containing the protein of interest were pooled. The pooled Mono Q fractions containing rp24 were finally purified by ultra filtration. Initially it was passed through a 50 kDa membrane (Millipore) followed by a 30 kDa membrane filter (Millipore) and centrifuged at 1000 x g for 5 min. The retentate of 30 kDa membrane filter was found to contain our protein of interest (by SDS-PAGE analysis and Western blotting of the same) in a concentrated form.

6. *Assay of sensitivity and specificity of ELISA*

The sensitivity and specificity of ELISA was assayed using the rp24 protein alone and in conjugation with other synthetic peptides obtained from the envelope of HIV-1 and HIV-2.

RESULTS AND DISCUSSION

The recombinant protein P24 was expressed in *E. coli* (BL21 DE3) after induction with IPTG (Fig 1). The localization of the recombinant protein was studied as described earlier. The protein rp24 was found to be present in the cytosol compartment as a soluble protein

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SDS-PAGE analysis of total cells of E. coli showing rp24 expression

Figure 1 *SDS-PAGE analysis of total cells of E. coli showing rp24 expression. The culture was induced with 1mM IPTG and the expression of protein in the total cell lysate was analyzed using 12 % gel. Lane 1: Marker proteins, Lanes 2 and 8: samples of before induction and Lanes 3-7: after induction samples.*

The immunoreactivity of rp24 was studied by Western blot analysis in the total cell lysates using anti-HIV positive and normal human sera (result not shown). Antibodies present in the HIV-positive sample recognized the recombinant protein (Fig 2). In a similar blot treated with normal human serum sample no bands corresponding to the rp24 lighted up, indicating that there was no non-specific interaction of the recombinant protein with serum components.

SDS-PAGE analysis of rp24 fractions

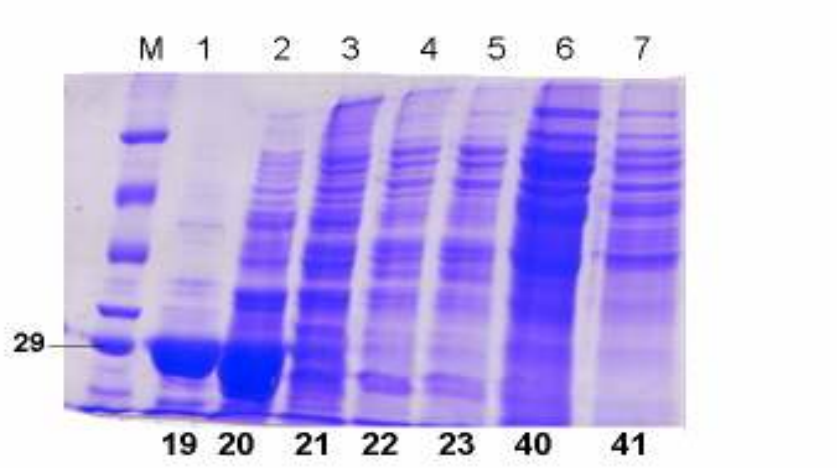

Figure 2 SDS-PAGE analysis of rp24 fractions from Q-sepharose column. The 100 ml supernatant of the cell lysate (using lysozyme) was loaded on Q-Sepharose matrix and the bound protein was eluted as described in the text. Various fractions of the column were collected and analyzed on a 12 % gel by SDS-PAGE. Lane 1: Marker proteins, Lanes 1-7: fractions (19-23 and 40, 41) of Q-Sepharose column. Desired protein was found to be present in the first 5 fractions (19-23) and the others were found to be impurities.

Since rp24 was expressed as a soluble protein, a simple scheme was developed for purification of rp24 from the cytosolic fraction of lysed *E. coli* without any *in vitro* refolding and renaturation steps. In the first set of experiments, binding conditions (pH and salt concentration) for the recombinant protein were ascertained in a batch binding experiment using Q-Sepharose. The rp24 protein was purified first by anion exchange (Q-Sepharose) column. First step efficiently captured the protein from soluble cytosolic fraction.

Hausdorf et al.[13] produced recombinant p24 with expression level of 15 % in *E. coli* and the yield of the reconstituted purified protein was 12 mg per liter in rich medium. Chaynet et al.[15] purified p17 and p24 with high level of expression (30 %) but with the recovery about 40 mg of the purified protein per liter of culture. Gupta et al. [14] also produced B- and C subtypes of p-17 from *E. coli* with low recovery; p17 (B-subtype) with 14.7 mg/L yield and p17 (C-subtype) with 7.7 mg/L. In addition, Sanchez et al.[20] introduced the purification of the natural 24 kDa (p24) with low recovery.

The column was developed as described and rp24 eluted at a salt concentration between 0.05 and 0.20 M (NaCl). This protein eluted from the Q-Sepharose column was 95 % pure. The protein was finally purified by gel filtration. The final yields of the rp24 protein were calculated to be in the range 20-30 mg per litre of culture on repeated experimentation (Fig 3).

SDS-PAGE of retentate and permeate

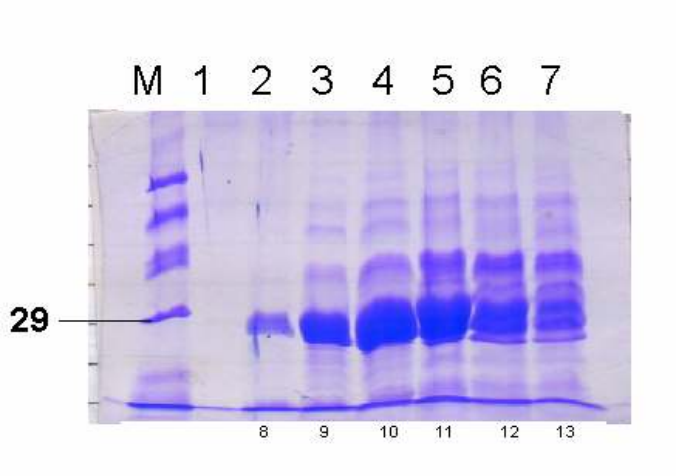

Figure 3 *SDS-PAGE of retentate and permeate obtained from gel filtration (50 and 30 kDa membranes). The Dialyzed sample (40 ml) was loaded on Q-Sepharose matrix and the bound protein was eluted as described in the text. Various fractions of the column were collected and analyzed on a 12 % gel by SDS-PAGE. Lane 1: Marker proteins, Lanes 1-6: fractions (8-13) of Q-Sepharose column. Desired protein was found to be present in all the selected fractions (8-13). Fractions 11, 12 and 13 were found to contain impurities that were removed in later stage by ultrafiltration.*

Optimization of the conditions for the use of only one anion exchange column followed by a simple gel filtration step has made the process amenable to reproducible results with high yield and thereby the process may be industrially useful (Fig 4).

SDS-PAGE analysis of samples showing finally purified protein

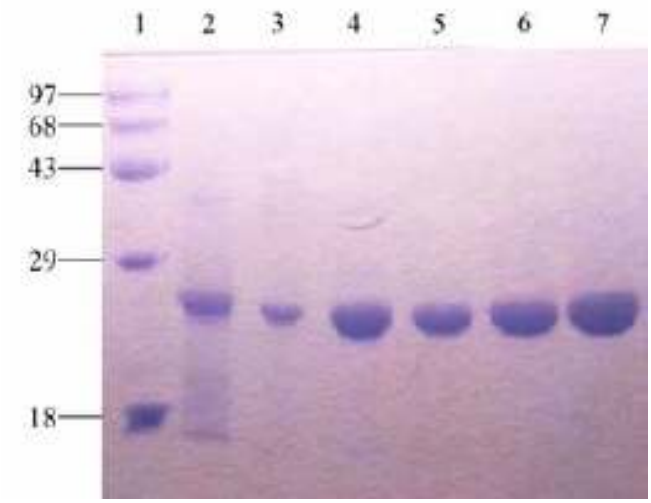

Figure 4 SDS-PAGE analysis of samples showing finally purified protein. Samples were first passed through a 50 kDa membrane filter followed by 30 kDa membrane. The retentate sample obtained was subjected to SDS-PAGE analysis using a 12 % gel. Lane 1: Marker proteins, Lane 2: permeate obtained from 30 kDa membrane, Lanes 3-7: purified and concentrated proteins obtained from the retentate of 50 kDa membrane filter.

The purified protein was used in ELISA. The overall results of the rp24 purification process have been shown in the Table 1.

Table 1
Summary of the results of rp24 purification process

Protein	Expression (%)	Final Purity (%)	Recovery (mg/L)
rp24	30	95	20-30

Development of ELISA

The purified rp24 was used in ELISA alone and in combination with other antigenic sequences from HIV-1 and HIV-2 for detection of anti-HIV antibodies in human serum and/or plasma. The results showed that rp24 alone, at all the three concentrations, detected HIV-positive samples with low sensitivity. These observations were not entirely unexpected, given the early window of appearance of anti-p24 antibodies in the course of HIV infection.

Table 2
ELISA Performance using Positive and Negative sera for HIV-1 and 2

1.937 PC	1.070 +	1.435 ++	1.773 ++	1.385 ++	0.749 +	1.866 +++	0.080 -	0.087 -	0.482 -	0.058 -	0.115 -
1.934 PC	1.488 ++	2.017 +++	1.936 +++	0.532 +	1.268 ++	1.562 ++	0.060 -	0.058 -	0.063 -	0.047 -	0.101 -
0.090 NC	1.462 ++	0.763 +	1.820 +++	0.681 +	1.290 ++	1.546 ++	0.078 -	0.056 -	0.033 -	0.018 -	0.041 -
0.095 NC	0.874 +	1.925 +++	1.008 ++	1.248 ++	1.414 -	0.071 -	0.038 -	0.064 -	0.048 -	0.027 -	0.085 -
1.371 ++	0525 +	1.609 ++	1.632 ++	1.740 ++	1.407 ++	0.046 -	0.115 -	0.036 -	0.046 -	0.056 -	0.008 -
1.888 +++	0.621 +	1.645 ++	1.637 ++	1.224 ++	0.725 +	0.086 -	0.054 -	0.040 -	0.058 -	0.020 -	0.006 -
1.468 ++	0.115 -	1.462 ++	0.392 ++	1.525 +	0.351 +	0.059 -	0.321 -	0.024 -	0.165 -	0.047 -	0.022 -
1.324 ++	0.923 +	1.580 ++	1.207 ++	1.499 ++	1.086 ++	0.057 -	0.044 -	0.047 -	0.074 -	0.071 -	0.004 -

+++ EQN= PC/7+1.5
= 1.776
++ EQN= PC/ 7+0.8
= 1.076
+ EQN= PC/ 7
= 0.276
EQN= PC/ 7-(PC/70)

+++ indicates a very strong positive reaction
++ indicates a strong positive reaction
+ indicates a positive reaction
- indicates a negative reaction.

The plate contains 96 wells.
The 1st and 2nd well contains PC (Positive control)
Wells 3 and 4 contains NC (Negative control)
Wells 5-46 contains HIV-1 positive samples.
Wells 47-51 contains HIV-2 positive samples.
Wells 52-86 contains NHS (Normal human serum)

Wells 87-96 contains CRP (Cross reactive positive samples like TB, HSB Ag, HCV, Syphilis but negative for HIV).

Moreover, the anti-p24 antibodies do not stay in circulation for long after the anti-envelope antibodies appear in the system. We then tested the performance of ELISA using rp24 at two different concentrations along with synthetic peptides from stretches of envelope proteins of HIV-1 (gp41) and HIV-2 (gp36) (Table 2). In this analysis with a relatively small sera panel, the ELISA test had a sensitivity and specificity of 100 %.

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