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# Cloning and Expression of Protease 2A from *Coxsakievirus B3*

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## ABSTRACT

**Protease 2A (2Apro) of *coxsackievirus B3 (CVB3)* plays a major role in viral replication. In case of infection, viral proteins are being synthesized from viral mRNA using host biosynthesis machinery. 2Apro of virus, after being synthesized, exhibits two critical functions, cleavage of viral proteins and breaking eukaryotic initiation factor 4G. The enzyme plays an essential role in viral replication and cellular damage. To understand pathogenicity of infection and also developing potent and selective inhibitors against picornavirus infection, it is necessary to prepare pure 2Apro enzyme. cDNA of 2Apro was synthesized using *in vitro* infection of permissive host through reverse transcription process and was cloned in pET22b(+). Since 2Apro is a toxic product, its expression will act on host before induction and damages the cells. For this reason, different hosts were checked and finally *BLR(DE3)pLysS,* which carries an extra-plasmid for lysozyme expression, that minimizes unwanted target protein products (leakage) was selected. By employing such expression system we could minimize the unwanted expression of 2Apro. Though it is not possible to avoide it, but seems negligible. Hence, this system is useful for expression of toxic proteins in sensitive hosts in order to prevent bacterial damage. The product was confirmed by SDS polyacrylamide gel electrophoresis and immunoblot analysis.** *Iran. Biomed. J. 9 (4): 149-153, 2005*

*Keyword*: Protease 2A (2Apro), Cloning, *Coxsackievirus*

## INTRODUCTION

Gene expression and replication of *coxsackie-virus B3 (CVB3)* are controlled by a complex cascade of proteolytic processing events which are mediated mostly by two viral gene products, protease 2A (2Apro) and 3C protease (3Cpro) [1]. 2Apro is a multifunctional polypeptide catalyzing an essential cleavage of the viral polyprotein at a tyrosine-glycine pair at the 1D-2A junction [1].

The 2Apro of picornaviridae are also responsible for the cleavage of the eukaryotic initiation factor 4G (formerly P220) leading to host cell protein synthesis shut-off [2-4]. Due to their unique protein structure and essential roles in viral replication, 2Apro and 3Cpro have been viewed as excellent targets for developing antiviral drugs [5, 6]. In recent years, considerable efforts have been made in development of antiviral compounds targeting these proteases [7]. Active 2Apro from *poliovirus coxsackievirus B4*, *Human rhino virus (HRV2)* and *HRV14* have been expressed in bacterial or mamalian cells and purified by several groups [8-12]. The availability of purified active recombinant 2Apro from *CVB3* , helps studying the effect of protease inhibitors against viral infection. In this study, recombinant 2Apro from *CVB3* was prepared which can be used for further investigation including antiviral drug designing.

## MATERIALS AND METHODS

***Materials.*** Materials were obtained from the following sources: HeLa cells, National cell Bank of Iran (NCBI C115); *CVB3* Nancy strain, American type tissue culture (Number VR-30); *E. coli BL-21, BLR(DE3), BLR(DE3)pLysS*, pET 22b(+) vector, Novagen (USA); RPMI, FCS, Agarose, Gibco (England); isopropanol chloroform, $CaCl_2$, Merck (Germany); random hexamer, revers transcriptase, tag polymerase, *EcoRI, NdeI*, T4 Ligase, Ampicilin, tetracycline, Roche Diagnostic (Germany); luria broth, Scharlau (Spain).

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***Viral RNA preparation.*** RPMI 1640 medium supplemented with 10% FCS was used for growth and maintenance of HeLa cell cultures. Cells at 80% confluency were infected with *CVB3* in a medium containing 1% FCS by $10^5$ plaque-formation units. Infected cells were collected after 24 h by centrifugation at 300 ×g for 10 min and total RNA was extracted by RNX solution.

***DNA recombinant technology.*** Extraction of plasmid, digestion, isolation, ligation, transformation, identification, PCR and so on were performed as described in standard literature [13]. cDNA was synthesized by reverse transcriptase M-MULV enzyme (Roche Diagnostic company manual, Germany). 2Apro cDNA was amplified by PCR using a set of forward and reverse primers. The forward primer contained *NdeI* restriction site and reverse primer had *EcoRI* site after stop codon.

***Construction of expression vector.*** In this reaserch, pET22b(+) was used as expression vector. 2Apro cDNA digested by *NdeI and EcoRI* restriction enzyme was ligated to the same digested site in the vector using T4 ligase to form pET-2Apro.

***Recombinant 2Apro expression.*** Competent *E. coli BLR(DE3)pLysS* cells were transformed with pET22b(+) expression vector containing 2Apro cDNA (pET-2Apro). *E. coli* cells were grown in shaker flasks at 37ºC, in LB broth containing 100 µg/ml ampicilin and 15 µg/ml tetracycline until OD = 1.0. Then, 1 mM of Isopropyl-Beta-D-Thio-galacto-pyranoside (IPTG) was added to the medium to induce 2Apro expression. In 0, 1, 2 and 3 hours after induction, samples were taken, cells were centrifuged at 3000 ×g for 5 minute and used for further studies.

***Production of rabbit antiserum against 2Apro.*** Immunization of rabbits was carried out as described elsewhere [14]. Due to the lack of purified 2Apro, rabbits were injected by *CVB3* as a source of 2Apro production.

***Immunoblot analysis.*** For characterization of expressed 2Apro protein, samples were lysed by addition of protein sample buffer 2x (100 mM tris-HCl pH 6.8, 200 mM dithiotheritol, 4% SDS, 0.2% bromo-phenol blue and 20% glycerol) and heated at 100˚C for 5-10 min.

Then, extracts were electrophoresed in SDS-14% polyacrylamide gel and transferred into a polyvinylidene fluoride (PVDF) membrane (Roche Diagnostic, Germany). PVDF sheet was blocked with 3% BSA in TBS-T solution (20 mm, tris-HCl pH 7.5; 150 mm, NaCl and 0.05% Tween 20). After blocking, the anti-2Apro polyclonal antibody (1:300 dilution in TBS-T buffer) was added for 1 h. A second incubation with HRP anti-rabbit Ig antiserum (1:1000 in TBS-T) was carried out. A third incubation of 5-10 min was done with diaminbanzine (DAB) solution (0.5 mg/ml DAB and 0.1% $H_2O_2$).

## RESULTS

***Molecular cloning of 2Apro.*** Total RNA was extracted from infected HeLa cells and cDNA was synthesized by reverse transcriptase enzyme. In order to clone DNA, restriction enzyme sites were introduced into primers. PCR product obtained was analyzed on agarose gel and 460 bp band was confirmed (Fig. 1). This fragment was inserted to pBluescript cloning vector and transferred to *E. coli* TOP10F strain for propagation. After cultivation, screening of transformant and plasmid extraction digestion with *EcoRI and NdeI* for confirmation was performed (Fig. 2). The positive clone was sequenced (Seqlab Company, England) using forward and revers primers and complete 2Apro cDNA was confirmed (Fig. 3). The pBluescript from positive clone used as template in PCR for subcloning.

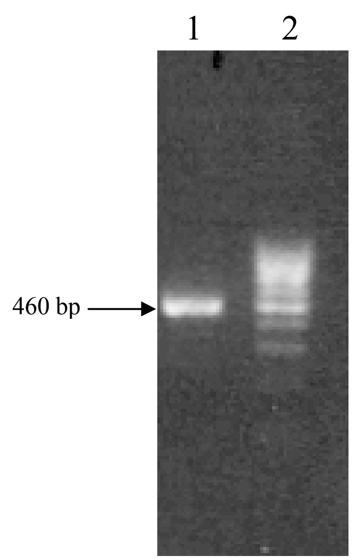


**Fig. 1.** Analysis of PCR product on Agarose gel. Lane 1, PCR product; Lane 2, 100 bp ladder.

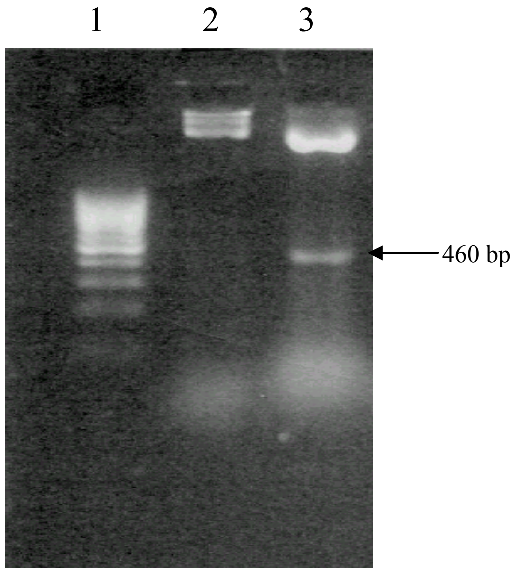


**Fig. 2.** Digestion product of PET22b(+) vector inserted with 2Apro. lane1, 100 bp ladder; lane 2, plasmid undigested; lane 3, plasmid digected.

***Expression of 2Apro.*** By digesting PCR product and pET22b(+) with *NdeI* and *EcoRI* seperately and eluting digests, they were ligated using T4 ligase to form pET-2Apro (Fig. 4). pET-2Apro recombinant vector was transferred to *E.coli BLR(DE3)pLysS* strain and transformant bacteria were grown in LB medium containing ampicilin at 37˚C and induced to express 2Apro with 1 mM-IPTG.

ACCCAT**ATG**GGCGCATTTGGACAACAATC
AGGGGCAGTGTATGTGGGGAACTACAGGG
TAGTAAATAGACATCTAGCTACCAGTGCT
GACTGGCAAAACTGTGTGTGGGAAAGTTA
CAACAGAGACCTCTTAGTGAGCACGACCA
CAGCACATGGATGTGATATTATAGCCAGA
TGTCAGTGCACAACGGGAGTGTACTTTTGT
GCGTCCAAAAACAAGCACTACCCAATTTC
GTTTGAAGGACCAGGTCTAGTAGAGGTCC
AAGAGAGTGAATACTACCCCAGGAGATAC
CAATCCCATGTGCTTTTAGCAGCTGGATTT
TCCGAACCAGGTGACTGTGGCGGTATCCT
AAGGTGTGAGCATGGTGTCATTGGCATTG
TGACCATGGGGGGTGAAGGCGTGGTCGGC
TTTGCAGACATCCGTGATCTCCTGTGGCTG
GAAGATGATGCAATGGAACAGTGA**ATT**CG
GACTA

**Fig. 3.** DNA sequencing of cloned signal sequence-containing cDNA fragment. Nucleotide sequence of cloned cDNA was according to nucleotide sequence in Gene Bank. *NdeI* (CATATG) and *EcoRI* (GAATTC) restriction sites have shown as underline and initiation codone (ATG) and stop codon (ATT) have presented as Bold.

Figure 5 shows the Coomassie blue staining of protein extracts from bacteria harvested at different time intervals after induction. The Figure shows a new band comparing to control with an apparent molecular mass of 16 kDa, which coincides with the expected size of 2Apro (16.6 kDa). The protein was confirmed by Western-blot analysis, using poly clonal antibody (Fig 6). The 16 kDa protein induced in bacteria reacted with antibodies prepared by injection of *CVB3* into female rabbit.

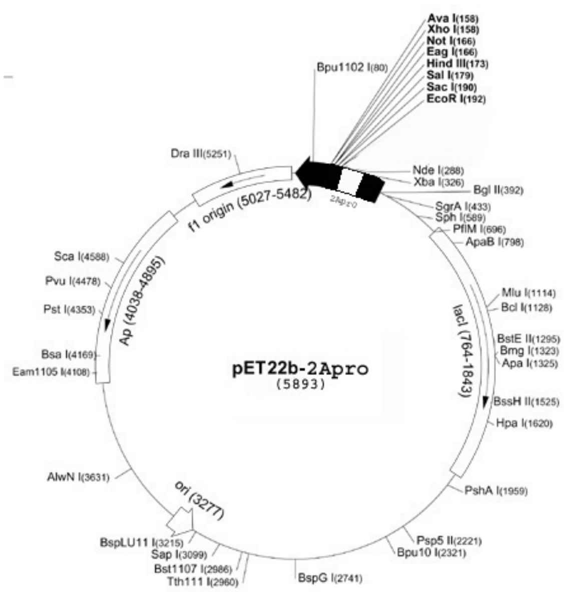


**Fig. 4.** Structure of expression vector pET22b (+).

## DISCUSSION

The picornavirus family encompass pathogens which are associated with several human infectious diseases including acute hepatitis, common colds and other upper respiratory tract infection [1]. *HRV2* and *coxsakieviruses* of this family are causative agents for common cold, meningitis and cardiomyopathy and despite of all developments, there is no vaccine or satisfactory antiviral therapy available for them, and also not for other viruses of the picornavirus family [1]. In search for a potential prophylactic treatment, it seems that 2A and 3Cpro encoded by these viruses represent attractive targets for antiviral therapy [15, 16].

As explained, 2Apro is a key enzyme involved in replication and infection of several entroviruses and obliteration of its activity can stop further viral proliferation. As a cysteine protease, it has been

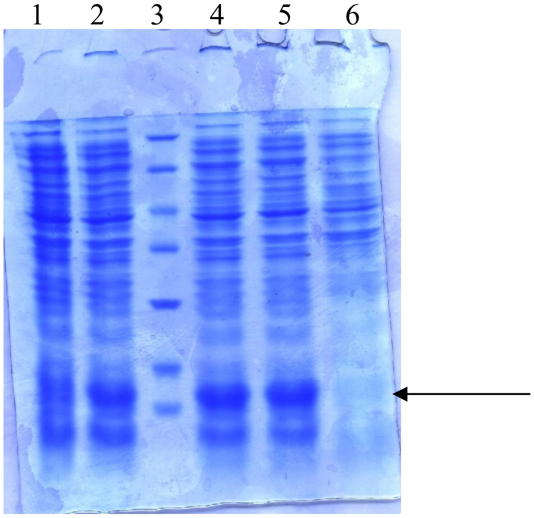


**Fig. 5.** SDS-PAGE of *E.coli BLR(DE3)pLysS*. Recombinant strains. Lanes: 1, bacteria with plasmide before induction; 2, bacteria with plasmid one hours after induction; 3, molecular weigh marker (116, 66, …, 18.4, 14.4 kilodalton); 4, bacteria two hours after induction; 5, bacteria three hours after inuction; 6, bacteria without plasmid.

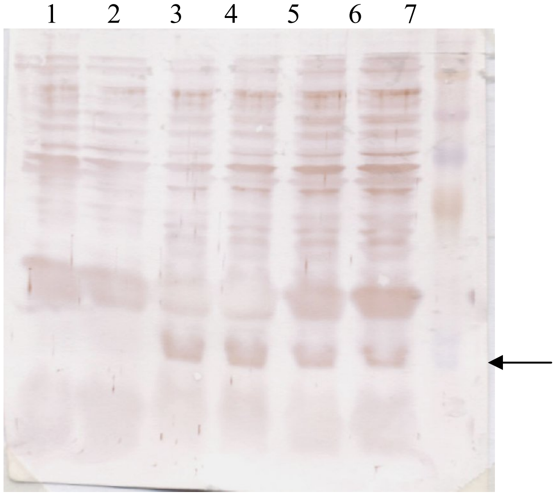


**Fig. 6.** Immunoblot analysis of 2Apro. Immunoblot analysis was done with rabbit antiserum obtained as indicated in methods. lanes: 1 and 2, bacteria without plasmid; 3, bacteria with plasmid three hours after induction; 4, two hours after induction; 5, one hours after induction; 6, before induction; 7, ribbon molecular weight marker (250, 160, 25, 15 and 10 kDa).

observed that 2Apro is sensitive to thiol alkylating reagents such as iodoacetamide and N-ethyl-maleimide [17]. Inhibition of 2Apro cleavage by activity of classic elastase-specific inhibitors has also been reported [17]. Hence, it seems that availability of pure 2Apro can greatly help to the development of potent and selective protease inhibitors against it, which can lead to prevent picornavirus infection.

In present study, we prepared cDNA from *CVB3*-2Apro and transfered it to an appropriate host. For this the vector was first transferred to *BL-21* and *BLR(DE3)*, but due to toxicity of 2Apro for the host there was a very sharp decrease in viability of the cells, as even without induction, there is always a minor expression of the target protein (leakage), hence we tried hosts which contain extraplasmid expression proteins, lysozyme, which in absence of induction inhibit polymerase activity. Therefore, with this unwanted expression of target gene is reduced [18] and by optimizing the gene expression condition, a proper source of pure 2Apro is obtained. It should be mentioned that due to unavailability of monoclonal antibody for precise detection of 2Apro protein band with no disturbance, we here used polyclonal antibody where other nonspecific bands also could be seen, but in other study the biological activity of this protein after purification was confirmed (unpublished data). Using pure recombinant 2Apro, it will be possible to search for 2Apro inhibitors and select potent inhibitors of viral replication both *in vitro* and *in vivo*.

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