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Cloning, Expression and Purification of the Truncated C-terminal Fragment of *Mycobacterium tuberculosis* HSP70 Gene in Prokaryotic System as a Tool for Vaccine Research

[1]*Seyyed Mahmoud Ebrahimi,* [1]*Majid Tebianian and* [2]*Habib Paykari*

[1]Department of Biotechnology,
[2]Department of Parasitology,
Razi Vaccine and Serum Research Institute, P.O. Box 31975/148, Karaj, Tehran, Iran

Abstract: New efficient vaccines against infectious diseases are in demand. Some important factors impeding the vaccine development are the poor immunogenicity and the MHC restriction of the immune responses to a number of antigens. The potential of heat shock proteins (HSPs) to function as adjuvant when fused to or co-delivered with protein antigens, make them attractive vaccine candidates. This study was designed to produce C-terminal domain of heat shock protein70, $HSP70_{359-610}$, as a genetic adjuvant. Therefore, the *Hsp70* gene was amplified by PCR from *Mycobacterium tuberculosis* (H37Rv), then inserted in pQE-30, prokaryotic expression vector. This recombinant protein, with a 6xHis- tag, was successfully over expressed in *Escherichia coli* strain M15. In order to obtain high yields of the recombinant HSP70 protein, inclusion bodies from bacterial cell lysates were solubilized, then recombinant protein was purified by Ni-NTA affinity chromatography under denaturing conditions, followed by urea gradient dialysis. This protein would be used as a potential candidate for genetic adjuvant and more immunological study is under study.

Key word: Adjuvant · HSP70 · *Mycobacterium tuberculosis* · Cloning · Expression

INTRODUCTION

Heat shock proteins (HSPs) are some of the most conserved proteins present in all prokaryotes and eukaryotes [1-3]. They undertake crucial functions in maintaining cell homeostasis.

From an immunological point of view, HSPs have attracted increasing interest, since they serve as carriers of antigens and effectively induce antigen-specific B and T cell responses without the need of adjuvant help [4-7]. These immunomodulatory functions of HSPs are based on various properties: (i) HSPs stimulate the production of chemokines which attract immunological cells; (ii) HSPs possess the ability to activate dendritic cells (DCs), thus initiating innate immune responses; and (iii) HSPs are capable of delivering peptides to major histocompatibility complex molecules for the priming of adaptive immunity.

The HSP70 family is one of the best studied among the HSPs and is endowed with crucial immunological functions because of their ability to interact with professional antigen-presenting cells through different receptors [8-11]. Many cytokines (interleukin-12, tumor necrosis factor alpha [TNF-α] and gamma interferon [IFN-γ] and CC chemokines are elicited by HSP70.

Microbial HSP70 (mHSP70) consists of three functionally distinct domains: an N-terminal 44 kDa ATPase portion (amino acids 1-358), followed by an 18 kDa peptide-binding domain (amino acids 359-494) and a C-terminal 10 kDa fragment (amino acids 495-610). Immunological functions of these three domains in stimulating monocytes and DCs have not been fully defined. However, the C-terminal portion (amino acids 359-610) was proven to stimulate the production of CC chemokines, interleukin-12 (IL-12), alfa tumor necrosis factor (TNF-α), NO and the maturation of Dcs [12]. A cytokine-stimulating epitope ($HSP70_{407-426}$) was identified in the peptide-binding groove of HSP70. It could activate and significantly enhance maturation of DCs stimulated by microbial HSP70 (mHSP70) or CD40L [13]. In addition, a cytotoxic cell-inducing function was demonstrated in the ATPase portion (amino acids 161-370) of mycobacterial HSP70 [14].

Corresponding Author: Seyyed Mahmoud Ebrahimi, Department of Biotechnology, Razi vaccine and serum research Institute, Karaj, Tehran, Iran, P.O. Box 31975/148, Tel: + 98 261 4570038-46

$HSP_{161-370}$ enhanced antigen-specific CTL responses via a $CD4^+$-T cell independent pathway. Many studies have described the ability of mHSP70s to enhance the immunogenicity of associated antigens [4, 15-18].

One major challenge in developing effective vaccines is to design a vaccine that can induce effective immune responses to the desired antigen with no or very limited side effects. Poor immunogenicity and MHC restriction hamper the potential of many candidate antigens [19]. The immunogenicity can be improved by using appropriate carriers and adjuvant molecules. HSPs are highly immunogenic and function as adjuvants that may play a crucial role in integrating innate and adaptive immunity.

Thus, our main strategy was to evaluate the adjuvant effect of the C-terminal domain of HSP70, $HSP70_{359-610}$ and to explore the possible mechanisms and effectiveness of selected fragment of the HSP70 family in exerting adjuvanticity in an animal model.

So, this study aimed at cloning, expression and purification of the truncated *M. tuberculosis* C-terminal $HSP_{359-610}$ in prokaryotic system, but further study will be needed to evaluate the adjuvant properties of this molecule in animal model by delivery system such as nanoparticles.

MATERIALS AND METHODS

PCR Amplication and Dna Cloning: The C-terminal domain of HSP70, $HSP70_{359-610}$, was synthesized by PCR using the genome of *Mycobacterium tuberculosis* (H37Rv) as a template.

The upstream primer (5' CGCGGATCCGAGGTGAA AGACGTTCT 3') contained a *Bam*HI site. The downstream primer (5' CCCAAGCTTCTTGGCCTC CCGGCCGTC 3') contained a *Hind*III site and without stop codon.

Polymerase chain reaction (PCR) was performed in a 50 µl mixture containing 5 µl of 10X reaction buffer with MgSO4 (2 mM), 4 µl of mixed dNTPs (2.5 mM each), 1 µl of each specific primer (10 pmol each), 0.5 µl of *pfu* DNA polymerase (2.5 u/µl) (Mannheim, Roche, Germany), 1 µl of template and 37.5 µl of nuclease-free water. The PCR program involved denaturation at 95°C for 3 min, followed by 5 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min. Then 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The resulting PCR products were then analyzed by 1.5% (w/v) agarose gel electrophoresis.

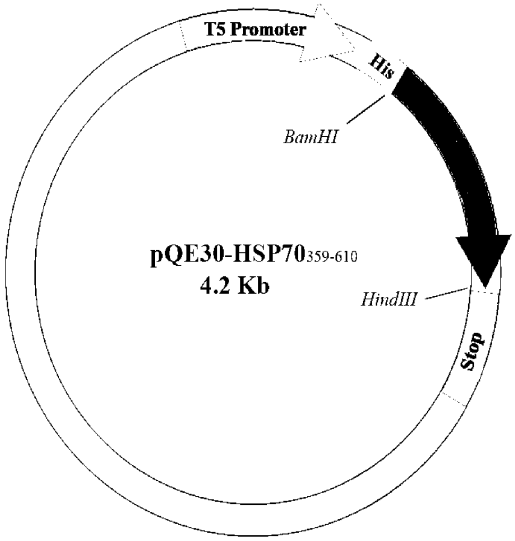

Fig. 1: Schematic representations of the recombinant expression vector; plasmid and and and pQE30-$HSP_{359-610}$ was constructed to express recombinant $HSP70_{359-610}$ protein.

Construction of Recombinant Plasmid PQE30-hsp70$_{359-610}$: The amplified fragment, $HSP70_{359-610}$, was gel-purified using high pure PCR product purification kit (Roche, German), according to manufacture protocol. The product was then double digested with *Bam*HI and *Hind*III restriction enzymes (Fermentas, Germany) and then ligated into the *Bam*HI / *Hind*III double digested pQE-30 expression vector (Qiagen, USA) using *T4* DNA ligase (Roche, Germany) to form recombinant expression vector pQE30-$HSP_{359-610}$ (Fig. 1).

E. coli strain DH5α was transformed with the ligated vector and transformants were selected on LB-agar plates containing 100 µg/ml ampiciline. Single colonies were selected and the sequence of the isolated plasmids was analyzed by the MWG Biotech Co. (Germany) to verify the presence of the correct and desired insert.

The procedures for small-scale preparation of plasmid, digestion with restriction enzymes, ligation and transformation all followed according to the manufacture protocols.

Expression of the Recombinant Hsp70$_{359-610}$ Protein *in E.coli*: Constructed pQE30-$HSP70_{359-610}$ was transformed into *E.Coli* host strain M15 (pREP4) (Qiagen, USA) competent cells. The cells were cultured in LB broth containing mixed 100 µg/ml ampicilin and 30 µg/ml kanamycin, then incubated at 37°C on a shaker incubator until optical density at 600 nm reached 0.6.

Then for induction of gene expression, the isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and subsequent propagation for 4 h at 37°C. Bacterial cells were collected by centrifugation and the cell pellets were further subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the expression of the recombinant proteins.

SDS-Page and Western Blotting: Protein samples were separated on 15 % SDS-PAGE according to the method of Laemmli [20]. Proteins were stained with Coomassie Brilliant Blue R-250 and a broad-range marker (Fermentas, Germany) used for the estimation of protein size.

For Western blotting, proteins in the 15% gel were transferred to polyvinylidene difluoride (PVDF) membrane. Then the immunoblotting carried out using penta-His HRP conjugate (Qiagen, USA), according to manufacture protocol. The protein bands were discovered by their exposure to Diaminobenzidine (DAB) substrate.

One-Step Purification of Recombinant Protein from the *E.coli* Lysate: The recombinant $HSP70_{359-610}$ protein was easily purified based on the fusion of 6xHis-tag at the N-terminus through the application of Ni-NTA affinity columns (Qiagen, Germany) under a denaturing condition. To purify the protein in a large scale, *E. coli* (M15) cells expressing the recombinant plasmid (pQE30-HSP70) were grown and induced in a 100 ml culture as above mentioned. The final bacterial cell pellet was resuspended in 8 ml of denaturing lysis buffer (6 M guanidine HCl, 20 mM sodium phosphate, pH 7.8, 500 mM NaCl). The cells were further lysed by sonication (15 s pulses at 15 s intervals for 6 times). After loading of the lysates on the Ni-NTA column and washing steps, recombinant protein was eluted with imidazole buffer (8 M urea, 20 mM Na2HPO4, 500 mM NaCl, 500 mM imidazole, pH 4.0). The protein was dialyzed in dialysis buffer containing 50 mM Tris and 1 mM EDTA for 48 h, with buffer changes every 12 h to remove the urea and imidazole. The purity of the recombinant protein was then evaluated on SDS-PAGE. To refold the purified denatured protein, dialysis was additionally performed in 500 ml of freshly made 0.01 M PBS containing gradient decreasing concentrations of 6, 4, 2, 1, 0.5 and 0 M urea in 5 mM Tris (pH 7.4) for 15 h at 4 °C.

RESULTS

PCR was able to amplify the desirable fragment (~820-bp; containing restriction site linker) of the truncated C-terminal fragment of the *Mycobacterium tuberculosis* $HSP70_{359-610}$ gene (Fig. 2).

Then the $HSP70_{359-610}$ gene was successfully cloned into the multiple cloning site region of pQE-30 expression vector.

The resulting plasmid was transformed into the *E. coli* strain DH5α. The positive clones were screened with colony PCR by the $HSP_{359-610}$ upstream and the downstream primers. The PCR products were confirmed to contain ~ 820-bp as expected by agarose gel electrophoresis.

Correct orientation was identified with the aid of the restriction analysis (RE) using *Bam* HI and *Hind* III enzymes. Restriction enzyme analysis of the clones gave the expected size of the two bands (~820 bp and 3.4 kb respectively) (Fig. 3).

The $HSP_{359-610}$ gene sequencing also showed 100% homology with the *Mycobacterium tuberculosis* H37Rv hsp70 gene (1075-1888 bp) deposited in Genbank with accession number of BX842573 (Data not shown).

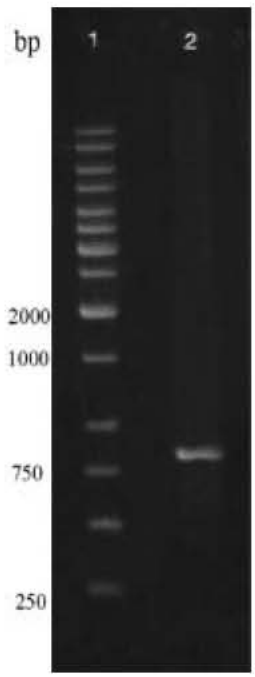

Fig. 2: Analysis of the PCR products on 1.5 % (w/v) agarose gel. Lane 1, contains 1kb ladder (fermentas, Germany); lane 2, contains $HSP70_{359-610}$ (~820bp)

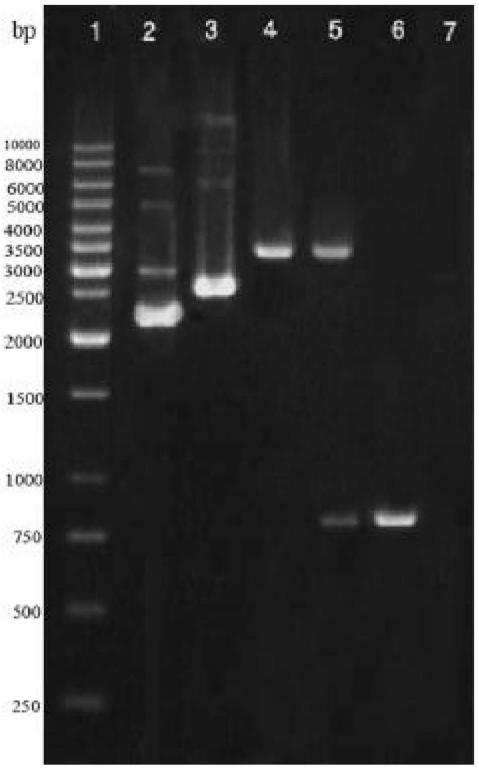

Fig. 3: PCR and restriction enzyme analysis of the constructed pQE30- HSP70 on 1.2 % (w/v) agarose gel. Lane 1, size marker 1Kb; lane 2, pQE30 plasmid without insert ; lane 3, pQE30 with insert (pQE30-HSP70) ; lane 4, pQE-30 plasmid (linerized by *Eco* RI enzyme) ; lane 5, pQE30-HSP70 double digested by *Bam* HI and *Hind* III enzymes; lane 6, PCR analysis of pQE30-HSP70 using specific primers; lane 7, negative control.

E. coli strain M15 was chosen as the expression host for the constructed recombinant plasmid. Based on the presence of six histidine [$(HIS)_6$] sequences at 5´-end of the multiple cloning sites (MCS) of pQE-30 expression vector, the 6xHis-HSP70 protein would have an estimated size of approximately 28 kDa on SDS-PAGE. After induction with IPTG, bacterial cells expressed the protein, which was detected in stained 15% SDS-PAGE as a ~28 kDa band (Fig.4).

For purification of the recombinant 6xHis- HSP70 protein, bacterial cells pellet recovered from the large scale culture was ultrasonicated and using the 6 M guanidine HCl, the inclusion bodies were dissolved. The expressed proteins were purified on the Ni-NTA columns and the ~28 kDa band corresponding to the purified fusion protein was observed in the SDS-PAGE gel (Fig. 4).

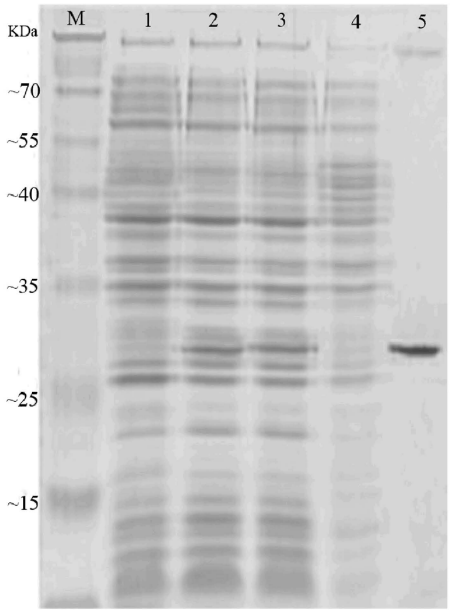

Fig. 4: SDS- PAGE analysis of *E. coli* strain M15 stained by Coomassie Brilliant Blue, showing the expression of $HSP70_{359-610}$ gene. lane M, broad-range marker (Fermentas, Germany); lane 1, before induction; lane 2, 1 h after induction; lane 3, 3 h after induction; lane 4, negative control; lane 5, purified expressed HSP70 protein.

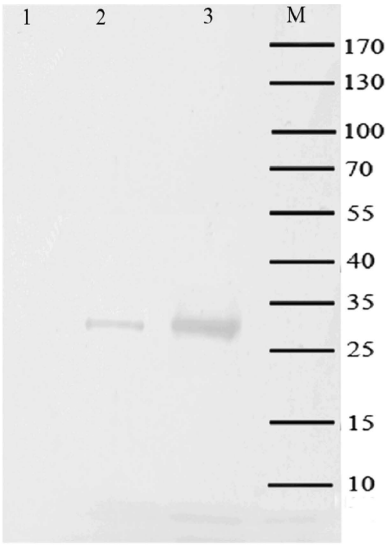

Fig. 5: Western blot analysis of expressed $HSP70_{359-610}$ protein on PVDF membrane using anti-penta-His antibodies. Lane M, Marker, Lane 1, negative control, lane 2: His- tag based expressed protein in the crude lysate ; lane 3, His-tag based purified protein.

For renaturing and refolding of purified protein, dialysis in gradient urea (at decreasing concentration) was accomplished. The concentration of the fusion protein was not measured.

For confirmation of protein expression, Western-blot analysis was performed on both expressed protein in the crude lysate and His-tag based purified protein, using anti-penta-His antibodies (Qiagen, USA).

After visualization with DAB substrate, corresponding band with the expected size was observed (Fig. 5).

DISCUSSION

Several adjuvants including microbial components have been evaluated for their ability to induce efficient immune responses in animal models as well as in preclinical/clinical studies. Moreover, new efficient vaccines against infectious diseases are in demand. Some important factors impeding the vaccine development are the poor immunogenicity and the MHC restriction of the immune responses to a number of antigens. The use of novel vaccine adjuvants or carrier proteins, which are known to enhance the immunogenicity of the subunit antigens and provide T-cell help, can circumvent these problems. The potential of heat shock proteins (HSPs) to function as adjuvants when fused to or co-delivered with protein antigens, make them attractive vaccine candidates [19, 21].

It was shown that the C-terminal fragment of HSP70 acted as a carrier in mice when fused to the malarial antigen EB200 (HSP70-EB200) and considerably induced MHC responses [22]. The effects of two truncated HSP70 molecules, N-terminal domain $HSP70_{1-360}$, amino acids 1-360 and C-terminal domain $HSP70_{359-610}$, amino acids 359-610, of mycobacterial HSP70 was evaluated on the potency of antigen-specific immunity generated by a hepatitis B virus (HBV) DNA vaccination and it was shown that only the $HSP70_{359-610}$-fused HBV DNA vaccination resulted in a significant increase in hepatitis B surface antigen (HBsAg)-specific humoral response, while the $HSP70_{1-360}$ -fused vaccine did not. Moreover, $HSP70_{359-610}$-fused DNA vaccine did not induce anti-HSP70 antibody [19]. In other studies, the effects of $HSP70_{359-610}$ on foot and mouth virus (FMDV) and Japanese encephalitis virus (JEV) in mice were evaluated separately and the HSP70 markedly enhanced both the humoral and cell-mediated immune responses [23,24].

Taking together, in this project the $HSP70_{359-610}$ gene was cloned, sequenced, expressed in a prokaryotic system using pQE-30 expression vector, the expressed $HSP70_{359-610}$ protein was purified and then confirmed by SDS-PAGE and Western blotting assay.

Further study will be needed to evaluate the adjuvant effect of the C-terminal domain of HSP70, $HSP70_{359-610}$ and to explore the possible mechanisms and effectiveness of selected fragment of the HSP70 family in exerting adjuvanticity in a animal model by delivery system mixed with subunit and recombinant agent antigens which are used as a vaccine.

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