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Cloning, expression and purification of the influenza A (H9N2) virus *M2e* antigen and truncated *Mycobacterium tuberculosis* HSP70 as a fusion protein in *Pichia pastoris*

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ABSTRACT

Due to its conservation, the extracellular domain of the influenza A M2 protein (M2e) has the potential for being applied as a recombinant vaccine candidate against a wide range of strains, though its immunogenicity may need to be improved. The occurrence of several post-translational modifications within the structure of M2 protein may affect its immunopotency for the induction of humoral immune response. Herein, to construct a recombinant M2e-based vaccine candidate with the appropriate structural conformation and immunogenicity the corresponding nucleotide sequence from an H9N2 influenza strain was fused to the N-terminus of the truncated *Mycobacterium tuberculosis* $HSP70_{359-610}$, as a potent adjuvant, and following its cloning into the pPICZαA plasmid the fusion gene was expressed in *Pichia pastoris* KM71H yeast. The secreted protein was then easily purified from the culture media, based on the presence of polyhistidine tag and used for the production of rabbit polyclonal antisera. This raised antisera could recognize the native M2e protein on the surface of H9N2 influenza virus-infected MDCK cells at a comparable level with the commercial H2N2-specific anti-M2 antibody, which was evidenced with immunofluorescence and cell–ELISA assays. These results not only re-emphasized on the conservancy of the M2e antigen, but also pointed towards the applicability of the M2e–$HSP70_{359-610}$ fusion protein for the induction of specific antibodies capable of binding to the native M2e antigen on the infected cells. Collectively, this study implied that purified M2e–$HSP70_{359-610}$ represents a promising vaccine candidate; however, its *in vivo* potency for the induction of protection remains to be evaluated.

Introduction

The threat of highly virulent avian influenza, such as H5N1 virus, brings out an urgent need to develop a universal influenza vaccine, which may provide cross-protection against different influenza virus strains [1]. The extra-domain of influenza M2 protein (M2e), which is almost completely conserved among all subtypes of influenza A virus especially H5N1 and H9N2 [2], is considered as a promising candidate target for the development of a broad-spectrum recombinant influenza A vaccine. Previous studies revealed that M2e-specific monoclonal antibody "14C2" restricted viral growth *in vitro* [3] and protected mice against the virus challenge in passive transfer experiments [4]. Several M2-based vaccines have been already proved to provide a successful protection against the challenges with homologous and heterologous influenza viruses, including H5N1 subtype [5–8] and among them some evidenced for the important protective role of M2e-specific antibodies. Accordingly, the M2-hepatitis B core (M2-HBc)[1] fusion particles protected the vaccinated mice in challenge experiments by inducing M2e-specific antibodies [6] and Jegerlehner et al. further indicated that this protection was mediated via antibody-dependent cell cytotoxicity [9]. It was also shown that passive administration of monkey anti-M2e sera could protect the mice against influenza challenge [10]. However, any potential investigation in order to enhance the immunogenicity of the M2e-based vaccines for the induction of better protective responses may be also appreciated. Several strategies have been introduced to increase the potency of the vaccines including antigen targeting to endoplasmic reticulum for rapid intracellular degradation [11], directing antigens to antigen-presenting cells

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[1] *Abbreviations used:* M2-HBc, M2-hepatitis B core; APCs, antigen-presenting cells; HSPs, heat shock proteins; HBsAg, hepatitis B surface antigen; ORF, open-reading frame; BMGY, buffered minimal glycerol; BMMY, buffered minimal methanol; BSA, bovine-serum albumin; HRP, horse-radish peroxidase; DAB, diaminobenzidine; IFA, incomplete Freund's adjuvant; MDCK, Madin-Darby canine kidney; PBS, phosphate buffered saline.

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(APCs) by their fusion to APC receptor ligands [12] and co-injecting cytokines/co-stimulatory molecules as molecular adjuvants [13]. In recent years, various heat shock proteins (HSPs) and molecular chaperones have been also proposed as potent adjuvants for stronger induction of immune response [14,15].

The antigenic nature of mycobacterial HSP70 not only allows it to be used as a vaccine candidate against mycobacterium, but also discusses for its application as a potential immunocarrier molecule under certain circumstances. Two major fragments of HSP70 protein have been already recorded that include an N-terminal 44-kDa ATPase domain ($HSP70_{1-358}$) and a C-terminal 28-kDa domain ($HSP70_{359-610}$), containing an 18-kDa peptide binding region (aa359–540) [16]. Li et al. showed that only the $HSP70_{359-610}$-fused hepatitis B virus DNA vaccine induced a significant increase in hepatitis B surface antigen (HBsAg)-specific humoral response [17]. This result argued in favor of the power of $HSP70_{359-610}$ as a potential immunocarrier and, thus, prompted us for its application towards the delivery of influenza A M2e protein in the context of a candidate recombinant vaccine.

Therefore, in the present study, we sought to construct a fusion plasmid harboring the extracellular domain of the influenza A M2 protein (M2e), which was fused to the N-terminus of the truncated HSP70 ($HSP70_{359-610}$) molecule. This chimeric sequence was heterologously expressed in *Pichia pastoris* yeast, purified and further analyzed as a recombinant fusion protein.

Materials and methods

PCR amplification and DNA cloning

The *M2e* gene (72 bp) was PCR amplified from the previously described pAED4-M2 plasmid template [18], which carries *M2* gene from avian influenza virus A/chicken/Iran/101/98 (H9N2), using the upstream (5′-CCGGAATTCATGAGTCTTCTAACCGAG-3′) and downstream (5′-CGCGGATCCATCACTTGAATCGCTGCA-3′) primers that harbored the EcoRI and BamHI restriction sites (underlined sequences), respectively.

PCR was performed in a 50 μl reaction mixture containing 10×buffer with 2 mM $MgSO_4$, mixed dNTPs (2.5 mM each), specific primers (10 pmol each), 1.2 U of pfu DNA polymerase (Mannheim, Roche, Germany) and 100 ng of pAED4-M2 plasmid as template. Amplification program was set as 95 °C for 3 min, which was followed by 5 cycles of 95 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, and then 30 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The resulting PCR products were then analyzed by 1.5% (w/v) agarose gel electrophoresis.

The C-terminal domain of HSP70 ($HSP70_{359-610}$) was isolated from the genome of *Mycobacterium tuberculosis* (H37Rv) by PCR and using the specific upstream (5′-CGCGGATCCGAGGTGAAAGACGTTCT-3′) and downstream (5′-GCTCTAGACTTGGCCTCCCGGCCGTC-3′) primers, harboring BamHI and XbaI sites (underlined sequences), respectively. The PCR parameters were similar to the *M2e* program, except the annealing temperature that was set at 63 °C for 1 min.

The amplified fragments, *M2e* and $HSP70_{359-610}$, were gel-purified using high pure PCR product purification kit (Roche, Germany), and digested with BamHI restriction enzyme separately. Digested products were again gel-purified and ligated by *T4* DNA ligase (Roche, Germany) to form *M2e–HSP70*$_{359-610}$ fusion construct. In this approach BamHI restriction site (GAATTC), encoding two extra amino acids of Gly and Ser separated two parts of the fusion construct.

Subsequently, using M2e upstream and $HSP_{359-610}$ downstream primers a PCR reaction with the annealing temperature of 59 °C for 1 min was performed on the *M2e–HSP70*$_{359-610}$ ligation mixture. This PCR product containing the inframe fusion of *M2e* and *HSP70*$_{359-610}$ genes was double digested and cloned into the EcoRI/XbaI multiple cloning site of the digested pPICZαA expression vector (Invitrogen, USA) to form the pPICMH plasmid (Fig. 1), so that the fusion protein would be expressed in a single open-reading frame (ORF) from the vector-derived ATG start codon at the beginning of the α-factor signal sequence to the TGA stop codon after the polyhistidine tag.

Recombinant vectors were purified from the transformed DH5α *Escherichia coli* cells under the selection of 25 μg/ml Zeocin antibiotics (Invitrogen, USA) and after restriction analysis were confirmed by sequencing reactions.

Transformation of P. pastoris KM71H and selection of transformants

Electrocompetent cells of *P. pastoris* KM71H were prepared according to the instructions of Invitrogen company [19]. Briefly, the cells were washed in ice-cold sterile water for several times and finally resuspended in ice-cold 1 M sorbitol. The recombinant fusion plasmid (~7 μg) was linearized with PmeI restriction enzyme (Fermentas, Lithuania) and transformed into competent *P. pastoris* cells (80 μl) by electroporation using a Micropulser system (Bio-Rad, USA) and the parameters of 1500 V, 25 μF and 5 ms pulse length in 2 mm cuvettes. Transformants were selected on YPDS plates containing 100 μg/ml Zeocin after 3 days of incubation at 30 °C and right colonies bearing the chromosomally integrated copies of *M2e–HSP70*$_{359-610}$, were selected by PCR amplification of purified genomic DNA and the application of M2e upstream and $HSP70_{359-610}$ downstream primer pairs.

Expression and purification of M2e–HSP70$_{359-610}$ *protein in P. pastoris*

The transformed yeast cells were cultured in a shaking flask containing 50 ml of buffered minimal glycerol (BMGY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base and 1% glycerol) until optical density at 600 nm reached 6. Cells were harvested by centrifugation, resuspended in 50 ml of buffered minimal methanol (BMMY) medium

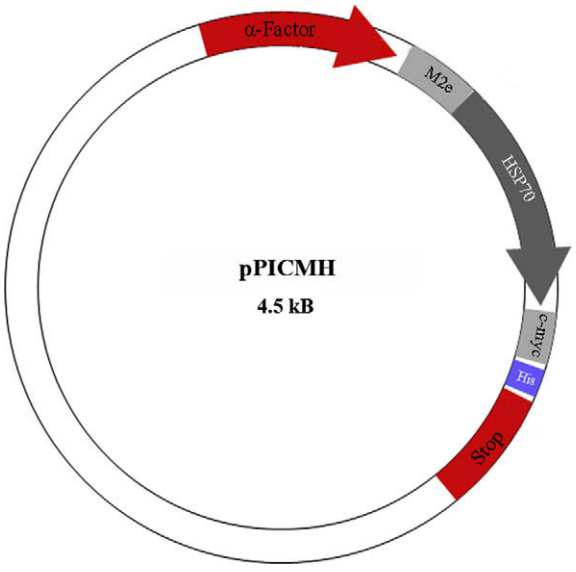

Fig. 1. Schematic representation of the recombinant expression vector; pPICMH plasmid, that encoded *M2e–HSP70*$_{359-610}$*–Cmyc–His* fusion as a secretary protein based on the presence of α-factor signal sequence.