The Development of A Bioassay Based on Heterologous Expression of M2 Ion-Channel Protein

Anggia Prasetyoputri[1*], Neti Yuliaty[1], Warda Tuharea[1], Alisin Febyanti[1], Bambang Sunarko[2] and Ines I. C. Atmosukarto[1]

[1]Research Centre for Biotechnology and [2]Research Center for Biology
Indonesian Institute of Sciences (LIPI), Indonesia

Abstract

Emerging resistant viral strains combined with the limited availability of antivirals in a pandemic scenario highlight the need for the development of novel influenza antivirals. A bioassay based on the M2 protein of influenza virus - a potential target for antivirals - was developed to screen endophytic microbial extracts. M2 can be synthesized using PCR, thus eliminating the need for the handling of infectious specimen. Following cloning of the M2 gene into a pET backbone, the resultant plasmid was transformed into BL21 (DE3) pLyss *E. coli* cells. Cultures of these cells were set up at 37°C following inoculation with a starter culture, to reach an OD at 600nm (OD_{600}) of 0.4-0.6. Once at the required OD, the culture was split in two aliquots and expression of the M2 protein was induced in one of the duplicates with the addition of isopropyl β-D-thiogalactopyranoside (IPTG). Bacterial growth was monitored at 60-minute intervals. Exogenous expression of the M2 protein has been reported to decrease host cells viability, resulting in lower OD_{600} values. Our results suggest that the M2 protein was expressed and that overexpression of this protein resulted in consistently lower OD_{600} values of induced cultures compared with that of uninduced cultures. Based on this principle, extracts can be screened for their ability to block M2 function as identified by increased OD_{600} values.

Keywords: influenza, M2, bioassay, gene synthesis, antiviral

---

[*]Corresponding author
Jl. Raya Bogor Km. 46, Cibinong 16911, Indonesia
Tel. +62-21-8754587 Fax. +62-21-8754588
E-mail. anggia.prasetyoputri@lipi.go.id; anggia.prasetyo@gmail.com

Introduction

The outbreak of avian influenza (AI), especially in the Southeast Asian region, has raised a concern for an influenza pandemic. Between 2003 and 2004 alone, nine Asian countries - China, Cambodia, Indonesia, Japan, Korea, Lao People's Democratic Republic (PDR), Thailand, Vietnam and Malaysia - were affected by the highly pathogenic H5N1 strain (Sims *et al*, 2005). In Indonesia alone, out of 167 confirmed cases, 138 of those were fatal (WHO, 2010). In addition, it is also feared that due to their high tendency to undergo mutations, human-to-human transmission of highly pathogenic AI will eventuate. Currently, available treatment for influenza are still limited to two classes of molecules, namely adamantanes (rimantadine and amantadine), which are M2 inhibitors (Hay, 1992; Lin *et al*, 1997; Wang *et al*, 1993; Wharton *et al*, 1994), and oseltamivir (Tamiflu) and zanamivir (Relenza), which are neuraminidase (NA) inhibitors (Calfee & Hayden, 1998; Moscona, 2005). The evidence of emerging resistant viral strains to both adamantanes (Hurt *et al*, 2007; Ilyushina *et al*, 2005) and NA inhibitors (Monto *et al*, 2006) have been reported. Additionally, with the threat of an imminent pandemic and limited availability of antivirals in a pandemic setting, there is an urgent need to develop new antivirals.

In order to find new lead molecules with antiviral activity against influenza, an assay with a specific target and appropriate design is required to screen the available extracts. The target protein in this scenario must be easily obtained, either in its native form or as a recombinant protein, and its expression easily detected. The influenza virus M2 protein essentially meets these criteria. As an

ion-channel protein, it is a potential target for therapeutics (Pinto & Lamb, 2006). M2 is a 97-amino-acid polypeptide that associates as homotetramers linked by disulfide bonds to form ion-channels (Holsinger & Lamb, 1991, Sugrue & Hay, 1991). It is a minor protein in influenza virus compared to hemagglutinin and neuraminidase but is found in abundance in the surface of virus-infected cells, and its sequence is highly conserved across influenza A virus strains. This protein is essential for viral growth (Takeda *et al*, 2002) as it plays an important role in viral entry and maturation, namely uncoating of virus and equilibration of the pH between the acidic trans-Golgi network and neutral cytoplasm (Hay, 1992; Sugrue & Hay, 1991). M2 activity can be inhibited by adamantanes, where viral replication is blocked in the presence of these inhibitors.

Its small size allows for total synthesis of M2 gene, thus eliminating the need to isolate cDNA from infectious virus. M2 can be expressed as recombinant protein in bacterial or yeast cells. There is evidence that overexpression of M2 in yeast can lead to reduced host viability indicated by slower growth rate of yeast cells expressing high levels of M2 (Kurtz *et al*, 1995). Growth impairment due to toxic effects of M2 expression was also observed in baculovirus-infected insect cells (Black *et al*, 1993) and *E. coli* cells (Guinea & Carrasco, 1994). This evidence forms the basis of the bioassay being designed, where it is expected that extracts containing molecules able to block activity of M2 protein will alleviate the growth of host cells, and the difference in growth rate can be easily detected using spectrophotometer.

This study aims to develop a bioassay based on heterologous expression of M2 protein to screen for microbial extracts for potential molecules with antiviral activity. Although resistance to adamantanes as the currently available M2 inhibitor have been reported, this class of drugs is still beneficial in the event of a pandemic (Moscona, 2005). Furthermore, there is evidence that the use of rimantadine in combination with neuraminidase inhibitor in vitro has synergistic effects against influenza (Govorkova *et al*, 2004), hence M2 inhibitor remains useful for combinatorial prophylaxis for influenza.

Materials and Methods

Total synthesis of M2 gene. The M2 gene sequence from H5N1 influenza virus was obtained from GenBank (accession number AY651379). The sequence used in this study has the same amino acid sequence as the consensus sequence of M2 gene (MSLLTEVETPTRNEWECRCSDSSDPL VVAASIIGILH**L**ILWILDRLFFKCIYRRLK YGLKRGPSTAGVPESMREEYRQEQQSA VDVDDGHFVNIELE). Codon optimization was conducted to ensure optimal expression in bacterial host. Total synthesis of M2 gene was done using PCR according to Young & Dong (2004), with a number of modifications. The first step of synthesis involved designing primers to cover the entire gene sequence, with two primers were designed to include restriction sites for BamHI at each end of the gene to facilitate cloning into plasmid. Several PCR reactions were conducted to yield short sequences that will form the full length of M2 gene. The second step involved amplification of pooled PCR products from the previous reactions to yield the full-length M2 gene sequence. Aside from Taq DNA polymerase, Pfu DNA polymerase was also used in the PCR reaction to minimize the occurrence of mutations. Following final amplification, DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma) and precipitated with ethanol. DNA pellet was then resuspended in endonuclease buffer and digested with 30 μl T7 endonuclease (New England Biolabs) at 37°C for one hour followed by 55°C for another hour. Digested mixture was separated on 2.5% agarose gel. The correct band was excised from gel and DNA was extracted using Qiagen gel extraction kit (Qiagen) according to manufacturer's instructions.

Construction of pET3a_M2 recombinant plasmid. M2 gene with the correct sequence was amplified by PCR (94°C [5 minutes]; 35 cycles of 94°C [45 seconds], 55°C [45 seconds], 72°C [45 seconds]; 72°C [5 minutes]) using the plasmid DNA as template, followed by digestion with *Bam*HI (Fermentas) (37°C for 1 hour). Digested product was purified with Qiagen PCR purification kit (Qiagen) and separated on 2%

agarose gel to confirm that M2 gene was digested. To facilitate sequencing, M2 gene was cloned into pGEMT-easy plasmid (Promega) and transformed using heat-shock method into DH5α *E. coli* (Merck) competent cells prepared by $CaCl_2$ method. Single white colonies were selected and cultured overnight at 37°C with shaking in Luria-Bertani (LB) broth (Hi-Media) supplemented with 100 μg/mL ampicillin (Sigma) (LB/Amp100). Plasmid DNA was isolated using alkali-lysis method and digested with *Bam*HI (37°C for 1 hour) to obtain clones containing M2 gene. Recombinant plasmids were sequenced to screen for mutations that may be present. Recombinant plasmid DNA containing the correct sequence of M2 was kept at -20°C until needed. M2 gene was sub-cloned into pET3a plasmid to facilitate expression in *E. coli* host. To prepare the plasmid, pET3a plasmid DNA (Novagen) was amplified by transformation into DH5α *E. coli* competent cells. Subsequently plasmid DNA was isolated and then digested with *Bam*HI (37°C for 1 hour), followed by treatment with Calf-Intestinal Phosphatase (CIP; Fermentas) (37°C for 30 minutes) to prevent re-coiling of plasmid prior to ligation. After separation on 1% agarose gel, DNA was extracted using Qiagen gel extraction kit (Qiagen). Cloning of M2 gene into pET3a backbone was conducted using Quick Ligase (Invitrogen) for 5 minutes at room temperature, followed by transformation (heat-shock method) into DH5α *E. coli* competent cells. Single colonies were selected and cultured overnight with shaking in LB/Amp100 medium. Plasmid DNA was isolated, digested with BamHI (37°C for 1 hour), and separated on 2% agarose gel to confirm the presence of M2 gene. To facilitate protein expression, pET3a_M2 plasmid DNA was then transformed into BL21(DE3) pLysS *E. coli* (Merck) competent cells, followed by culturing in LB agar medium supplemented by ampicillin (100mg/ml) and chloram-phenicol (34mg/ml) (LA/Amp/chlor). The presence of M2 gene was again confirmed prior to induction study by digesting isolated pET3a_M2 plasmid DNA with *Bam*HI following the above steps.

Induction and confirmation of M2 protein expression. Induction study was done using different volumes of LB/Amp/chlor, namely 100 ml and 40 ml (in Erlenmeyers) and 1 ml (in 24-well plate) to check for any effects different culture volumes may have in final OD_{600} values. A single colony of BL21(DE3) pLysS *E. coli* containing pET3a_M2 was cultured overnight at 37°C with shaking in 5ml of LB/Amp/chlor medium as a starter culture, following which fresh LB/Amp/chlor medium was inoculated with starter culture (1 in 100 v/v) and grown to reach an OD_{600} of 04-0.6, measured with spectrophotometer (Beckman type DU 650). After the desired OD was achieved, the culture was split in two separate flasks or wells. This would result in two 50 ml cultures from a 100 ml culture, and two 20 ml cultures from a 40 ml culture. One ml of culture was sampled and reserved for control. To induce M2 protein expression, isopropyl β-D-thiogalactopyranoside (IPTG; Merck) was added to the culture (1mM final concentration) and then it was incubated at 37°C with shaking. After-wards, 1 ml aliquots of both uninduced and induced cultures were sampled at 15 and 30 minutes, centrifuged, and discarded the supernatant. Pellets were resuspended in loading buffer, boiled at 95°C for 5 minutes, and run on 20% acrylamide gel. The gel was then used for Western blot analysis using anti-M2 antibody (anti-M2 ab5416, Influenza A Virus M2 Protein antibody [14C2]).

Induction study in BL21 (DE3) pLysS *E. coli* cells. A starter culture of pET3a_M2 in BL21(DE3) pLysS *E. coli* was set up overnight at 37°C to inoculate fresh LB/Amp100 medium. Once at the required OD, the culture was split in two aliquots. Cultures with starting volume of 100 ml and 40 ml will be split into two 50 ml and 20 ml cultures, respectively, while 1 ml of culture was added to each well on 24-well plate. IPTG at 1 mM final concentration was added to each of the duplicate cultures to induce M2 protein expression. Bacterial growth was monitored based on the OD_{600} values at 60-minute intervals, comparing between the induced (IPTG added) and uninduced cultures.

Results and Discussion

M2 gene was successfully synthesised by PCR, resulting in PCR product of 294 base pairs (bp). M2 gene was also successfully cloned into pGEMT-easy (Promega) backbone and approximately 50 clones were obtained confirmed by *Bam*HI digestion to contain M2 insert, some of which were sequenced to screen for mutations. Sequencing results confirmed that the method of synthesis used yielded M2 gene that was prone to mutations as all clones sequenced exhibit at least one mutation. Using *Pfu* DNA polymerase with better proof-reading capability than *Taq* DNA polymerase in the PCR reactions did not have significant effect in reducing the occurrence of mutations. Digestion with T7 endonuclease was also expected to minimize mutations as this enzyme is able to cut double-stranded DNA that have substitutions, insertions or deletions within. However, the result demonstrated that this digestion step was not sufficient in reducing mutations as mutations still occurred in digested PCR products.

One clone exhibited a mutation that was able to be corrected by site-directed mutagenesis, yielding an M2 gene that had no change in the reading frame. This M2 gene was then successfully cloned into pET3a (designated pET3a_M2) and M2 gene presence was confirmed following digestion with *Bam*HI.

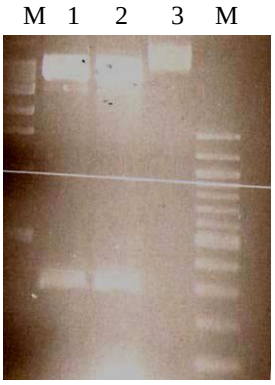

Figure 1. pET3a_M2 digested with *Bam*HI (Lanes 1-2: pET3a_M2/*Bam*HI; lane 3: uncut pET3a_M2)

To establish a bioassay based on heterologous expression of M2, pET3a_M2 recombinant plasmid was incorporated via transformation into BL21 (DE3) pLysS *E. coli* cells. These cells have the appropriate genetic background to support expression of proteins that are toxic in nature. M2 protein expression was induced by addition of IPTG and OD_{600} values were monitored at 60-minute intervals. It was reported that induction of M2 protein expression resulted in lysis of *E. coli* cells due to alterations in membrane permeability (Guinea & Carrasco, 1994). The results showed that M2 protein expression did result in reduced OD_{600} values of BL21 (DE3)-pLysS *E. coli* culture containing pET3a_M2 induced by addition of IPTG. Consistent results were achieved following at least two repeats of each induction study with different clones and culture volumes. A sample result of OD_{600} values can be seen in Table 1.

Table 1. The effects of induction of M2 protein expression on host cells viability

Culture	**Starting OD_{600}**	**60 minutes following induction**	**120 minutes following induction**
pET 3a_M2 clone 1 uninduced (control)	0.4370	0.8487	1.1237
pET 3a_M2 clone 1 induced		0.5249	0.5389
pET 3a_M2 clone 2 uninduced	0.4610	0.8850	1.1913
pET 3a_M2 clone 2 induced		0.5434	0.5573

Culture volume : 50 ml
IPTG concentration : 1 mM

M2 protein expression was confirmed using Western blot analysis using anti-M2 antibody, where M2 was expressed at the earliest 30 minutes following induction by IPTG (Figure 2). This result showed that eventhough the M2 gene was synthesised and not of viral origin, it was still able to be produced following induction using IPTG at the dose used (1 mM).

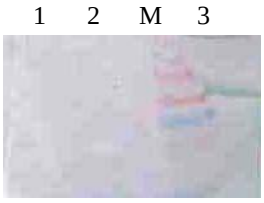

Figure 2. Western blot result of M2 protein expression (M: marker; 1: uninduced culture; 2: induced culture [15 minutes]; 3: induced culture [30 minutes])

In addition, transformants stored in glycerols at -40°C were also re-tested to ensure that long-term storage had no or little effect on the efficiency of M2 protein expression. It was found that the induction result using transformants that have been stored in glycerol for more than four weeks did not display the same trend as fresh transformants. Induction study results on a number of transformants that have been stored in glycerol at -40°C can be seen in Table 2. Therefore, screening will be conducted using only freshly transformed BL21 (DE3) pLysS *E. coli* cells and all stocks will be rejuvenated every 4 weeks.

Table 2. The effect of long-term storage on host viability following induction of M2 expression

Culture (all in glycerols)	**Starting OD_{600}**	**60 minutes induction**
pET3a_M2 1 clone 1 Uninduced	0.6019	1.0421
pET3a_M2 1 clone 1 Induced		1.0564
pET3a_M2 1 clone 2 Uninduced	0.6485	1.0764
pET3a_M2 1 clone 2 Induced		1.0401
pET3a_M2 1 clone 3 Uninduced	0.4982	0.8206
pET3a_M2 1 clone 3 Induced		0.8249

Induction study was done in a number of different volumes. For this comparison, the OD_{600} values were read at 30-minute intervals to be able to distinguish the difference in values between each volume. In terms of culture volume, there was no significant difference in host viability following induction of M2 expression in cultures of varying volumes (Table 3).

Table 3. The effect of culture volume on host viability following induction of M2 protein

Culture of pET3a_ M2 clone 1	starting OD_{600}	30 min induction	60 min induction	90 min induction
Uninduced	0.4707	0.7867	1.0150	1.2309
Induced 100 mL		0.5607	0.5821	0.5999
Induced 50 mL		0.5591	0.5918	0.6021
Induced 20 mL		0.5513	0.6046	0.6268
Uninduced	0.4492	0.7553	0.9765	1.1702
Induced 20 mL		0.5573	0.5807	0.6007
Uninduced plate #1		0.6132	0.6616	0.7189
Induced plate #1		0.5548	0.5677	0.5572
Uninduced plate #2		0.6031	0.662	0.7326
Induced plate #2		0.5455	0.5529	0.576

Comparing the results between larger cultures and those in 24-well plate, it can be seen that the difference in OD_{600} values between induced and uninduced cultures in general was easier to be distinguished in cultures of larger volumes. Therefore, the conditions for screening using 24-well plate still need to be optimized to ensure significant differences can be noted between induced and uninduced cultures. The results in Table 3 suggested that incubation time following induction should be at least 60 minutes to be able to note a difference in OD_{600} values between induced and uninduced cultures, eventhough the M2 protein was already expressed after 30 minutes following induction.

Conclusion

The bioassay based on the heterologous expression of M2 protein was able to be established. Induction of M2 expression was

able to cause a decrease in the host cells' viability, resulting in lower OD600 values in induced cultures compared to that in uninduced cultures. Therefore, this bioassay looks promising to be used to screen microbial extracts, although some optimization is needed to be able to do so in 24-well plate format.

References

Black RA, Rota PA, Gorodkova N, Klenk HD, & Kendal AP. 1993. Antibody Response To The M2 Protein Of Influenza A Virus Expressed In Insect Cells. *J Gen Virol* 74: 143-146.

Calfee DP & Hayden FG. 1998. New Approaches To Influenza Chemotherapy: Neuraminidase Inhibitors. *Drugs* 56(17): 537-553.

Govorkova EA, Fang H-B, Tan M, & Webster RG. 2004. Neuraminidase Inhibitor-Rimantadine Combinations Exert Additive And Synergistic Anti-Influenza Virus Effects In Mdck Cells. A*ntimicrobial Agents and Chemotherapy* 48(12): 4855-4863.

Guinea R & Carrasco L. 1994. Influenza Virus M2 Protein Modifies Membrane Permeability In *E. coli* Cells. *Febs Letters* 343: 242-246.

Hay AJ. 1992. The Action of Adamantanamines Against Influenza A Viruses: Inhibition of The M2 Ion Channel Protein. *Semin Virol* 3: 21-30.

Holsinger LJ & Lamb RA. 1991. Influenza Virus M2 Integral Membrane Protein Is Stabilized By Formation of Disulfide Bonds. *Virology* 183: 32-43.

Hurt AC, Selleck P, Komadina N, Shaw R, Brown L, & Barr IG. 2007. Susceptibility of Highly Pathogenic A(H5n1) Avian Influenza Viruses To The Neuraminidase Inhibitors and Adamantanes. *Antiviral Research* 73(3): 228-231.

Ilyushina NA, Govorkova EA, & Webster RG. 2005. Detection of Amantadine-Resistant Variants Among Avian Influenza Viruses Isolated In North America And Asia. *Virology* 341: 102-106.

Kurtz S, Luo G, Hahnenberger KM, Brooks C, Gecha O, Ingalls K, Numata K-I, & Krystal M. 1995. Growth Impairment Resulting From Expression of Influenza Virus M2 Protein In *Saccharomyces Cerevisiae*: Identification of A Novel Inhibitor of Influenza Virus. *Antimicrobial Agents and Chemotherapy* 39(10): 2204-2209.

Lin T-I, Heider H, & Schroeder C. 1997. Different Modes of Inhibition by Adamantane Amine Derivatives and Natural Polyamines of The Functionally Reconstituted Influenza Virus M2 Proton Channel Protein. *Journal Of General Virology* 78: 767-774.

Monto AS, Mckimm-Breschkin JL, Macken C, Hampson AW, Hay A, Klimov A, Tashiro M, Webster RG, Aymard M, Hayden FG, & Zambon M. 2006. Detection Of Influenza Viruses Resistant To Neuraminidase Inhibitors In Global Surveillance During The First 3 Years Of Their Use. *Antimicrobial Agents and Chemotherapy* 50(7): 2395-2402.

Moscona A. 2005. Neuraminidase Inhibitors for Influenza. *New England Journal of Medicine* 353(13): 1363-1373

Pinto LH & Lamb LA. 2006. The M2 Proton Channels of Influenza A and B Viruses. *The Journal of Biological Chemistry* 281(14): 8997-9000.

Sims LD, Domenech J, Benigno C, Kahn S, Kamata A, Lubroth J, Martin V, & Roeder P. 2005. Origin and Evolution of Highly Pathogenic H5n1 Avian Influenza In Asia. *Veterinary Record* 157: 159-164.

Sugrue Rl & Hay AJ. 1991. Structural Characteristics of The M2 Protein of Influenza A Viruses: Evidence That It Forms A Tetrameric Channel. *Virology* 180: 617-624.

Takeda M, Pekosz A, Shuck K, Pinto LH, & Lamb RA. 2002. Influenza A Virus M2 Ion Channel Activity Is Essential For Efficient Replication In Tissue Culture. *Journal of Virology* 76(3): 1391-1399.

Wang C, Takeuchi K, Pinto LH, & Lamb RA. 1993. Ion Channel Activity of Influenza A Virus M2 Protein: Characterization of The Amantadine Block. *Journal of Virology* 67(9): 5585-5594.

Wharton SA, Belshe RB, Skehel JJ, & Hay AJ. 1994. Role of Virion M2 Protein In Influenza Virus Uncoating: Specific Reduction In The Rate Of Membrane Fusion Between Virus and Liposomes By Amantadine. *Journal of General Virology* 75: 945-948.

WHO. 2010. Avian Influenza - Situation In Indonesia - Update 2. http://www.who.int/csr/don/2010_07_22/en/index.html. (Accessed: July 24, 2010).

Young L & Dong Q. 2004. Two-Steps Total Gene Synthesis Method. *Nucleic Acids Research* 32: 759.