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BIO-FUNCTIONAL ENHANCEMENT OF PLANT BASED-HAEMAGGLUTININ BY FUSION WITH IgMFc

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SUMMARY

The creation of recombinant oligomeric haemagglutinin proteins from A/H5N1 virus is a new concern for many scientists. In this study, the gene encoding the haemagglutinin protein (H5TG) derived from virus A/duck/Vietnam/TG24-01/2005 was fused with three different motifs (GCN4pII, GCN4pII-IgMFc and GCN4pII-ELP-IgMFc) in order to form three recombinant proteins (trimeric H5TGpII, oligomeric H5TGpII-IgMFc and ELPylated oligomeric H5TGpII-ELP-IgMFc, respectively) for enhancing protein expression, bio-function and purification of H5TG. These H5TG fragments have been attached to expression cassettes in the pCB301 shuttle vector, transiently expressed in *Nicotiana benthamiana* by agroinfiltration, then the protein expression was confirmed by SDS-PAGE and Western blot analysis. The results showed that the expression level of the H5TGpII-ELP-IgMFc protein in plant raw extract was stronger than that of two other proteins analyzed in this paper. Evaluation of bio-function showed that the haemagglutination titre (HA titre) of the total solution protein extract containing H5TGpII-IgMFc protein was highest (64 HAU) compared to that containing two remaining proteins (8 HAU). Subsequently, the H5TGpII and H5TGpII-IgMFc proteins were purified by immobilized metal ion affinity chromatography (IMAC), while the H5TGpII-ELP-IgMFc protein was purified using membrane-based Inverse Transition Cycling (mITC). The oligomer state of the purified proteins was then determined by non-reducing SDS-PAGE. The haemagglutination assay analysis of purified proteins showed that the lowest protein amount causing erythrocyte agglutination (1 HAU) of the H5TGpII-IgMFc protein was 0.06 µg and lower four times than that of H5TGpII and H5TGpII-ELP-IgMFc proteins (both of them were 0.24 µg). This indicates that the fusing of the GCN4pII-IgMFc motif into the H5TG protein gives the stronger bio-function than the fusing of two remaining motifs into this protein. This result opens up the applicability of the IgMFc for generation of oligomer proteins to enhance the bio-function of target proteins in the study of recombinant vaccine production.

Keywords: *assay, haemagglutinin, haemagglutination, IMAC, mITC, oligomer*

INTRODUCTION

Outbreaks of highly infectious and dangerous illnesses such as influenza A viruses in south-east Asia and especially in Vietnam (44 million birds, amounting to approximately 17.5% of the poultry population), have a strong influence on poultry production. Adaptation to a new host is facilitated by the high genetic flexibility of influenza A viruses. Influenza A

viruses belong to the family Orthomyxoviridae. Their genome includes a single-stranded negative sense RNA virus with eight segments, each encoding 1–3 structural and nonstructural proteins (Sfakianos, 2006). Haemagglutinin (HA) is coded by RNA segment 4, is the major envelope glycoprotein that initiates infection by binding to sialic acid-containing cell receptors and by inducing membrane fusion and viral polymerase that mediates transcription and replication of the viral genome. In addition, HA contains antigenic sites recognized by the host immune system, cleavage sites cleaved by host proteases (Klenk, 2015). HA is co-translationally translocated across the rough endoplasmic reticulum membrane (RER) and forms a precursor protein, a non-covalent homo-trimer (called HA0). Signal peptide (17 amino acids) cleavage and N-linked glycosylation occur during the co-translational process. Each resulting monomer has a molecular mass of approximately 60kDa for the unglycosylated form, and its molecular mass increases depending on the number and complexity of N-glycans for the glycosylated form with 549 amino acid residues. HA0 is transported through the Golgi complex to the plasma membrane and cleaved by cellular proteases to yield HA1 (327 amino acids) and HA2 (222 amino acids) (see review Sriwilaijaroen and Suzuki, 2012).

Vaccination is the most economically feasible approach to reduce illness and death from outbreaks (Gerhard, 2001). HA is the most important antigen to induce immunity and target to develop influenza candidate vaccines of influenza A viruses. The development of effective vaccines combined with low cost production against Influenza A viruses is a general important research goal. Vaccines to combat influenza A virus are conventionally produced in embryonated chicken eggs (Gregersen *et al.*, 2011), mammalian cells (Du *et al.*, 2011) which have been referred to be technically complex, slow and expensive. Haemagglutinin-based subunit vaccines produced in plants for example *Nicotiana benthamiana* as alternative expression systems become the focus of many researchers worldwide in recent years (Floss and Conrad, 2012; Kalthoff *et al.*, 2010; Shoji *et al.*, 2009). Plant-derived HA trimers elicited potentially neutralizing antibodies interacting with both homologous virus-like particles from plants and heterologous inactivated influenza A virus (Phan *et al.*, 2013).

The elastin-like polypeptides (ELP) fusion technology provides many advantages in the large-scale production of biologicals including increased yields, simple downstream processing by inverse transition cycling and biocompatibility (Floss *et al.*, 2010). The benefit of ELPylation has also been tested for HIV-1 antigens (Floss *et al.*, 2008 and 2009), TNFa nanobodies (Conrad *et al.*, 2011), human interleukin and spider silk protein (Patel *et al.*, 2007; Scheller *et al.*, 2004). ELP fusion can enhance the yield of heterologous proteins by between two- and 100-fold. The basis advantages of ELP may lie in the lesser susceptibility of ELPylated proteins to plant proteases (Zhang *et al.*, 1996) or hydrolysis (Raucher and Chilkoti, 2001). ELPylation of trimeric HA does not influence the trimerization. Strong expression enhancement in plants caused by ELPylation was demonstrated for trimerized H5-ELP (Phan *et al.*, 2013).

IgM is an alternative candidate for fusion protein technology, especially vaccine development since it forms natural polymers that binds C1q and TRIM2, is involved in antibody subclass switching, shapes the ensuing immune response and has been shown to be an excellent natural adjuvant in vaccines. IgM has the additional advantage of engaging unique receptors on B, T (CD4þ/CD8þ) and NK lymphocytes that may stimulate memory responses required by efficacious vaccines) (for review see Czajkowsky *et al.*, 2012). The Fc region of IgM is of outstanding interest because its structure, oligomerization, and effector protein binding clearly differs from other Ig Fc regions. The Fc part of IgM is composed of three Ig domains: Cμ2 (C337), Cμ3 (C414), and Cμ4 and an additional C-terminal tail piece (tp)

(C575). The Fc part of IgM is covalently linked in the Cμ2, Cμ3, and Cμ4 (tp) domains by inter-chain disulfide bridges (Müller *et al.,* 2013). Fc-tagged HAs are potential candidates for gene-to-vaccine approaches to influenza vaccination. Recently, the human immunoglobulin Fc domain was fused with the haemagglutinins (HAs) of human H1 and H3 influenza viruses and avian H5 influenza virus to produce as recombinant fusion protein from baculovirus-infected insect cells (Loureiro *et al.*, 2011). Recombinant HA-human immunoglobulin Fc domain (HA-HuFc) proteins as glycosylated oligomer HAs of the anticipated molecular mass, agglutinated red blood cells, were purified on protein A, and were used to immunize mice in the absence of adjuvant. Immunogenicity was demonstrated for all subtypes, with the serum samples demonstrating subtype-specific haemagglutination inhibition, epitope specificity similar to that seen with virus infection, and neutralization (Loureiro *et al.,* 2011).

In this study, to generate oligomeric haemagglutinins, trimeric haemagglutinin (H5TGpII) was fused with different motifs: IgMFc and ELP-IgMFc, then these genes were inserted in the pCB301 vector under the control of CaMV35S promoter. The expression vectors were transformed via *Agrobacterium tumefaciens* transiently into *Nicotiana benthamiana*. Five days after agro-infiltration, the expression of haemagglutinin proteins in tobacco leaf were detected by Western blot. Total soluble proteins in raw extracts containing haemagglutinin proteins were then bio-functional tested by haemagglutination assay. For purification of haemagglutinin proteins (H5TGpII and H5TGpII-IgMFc), Immobilized metal ion affinity chromatography (IMAC) was used. Membrane-based Inverse Transition Cycling (mITC) method was carried out to purify H5TGpII-ELP-IgMFc. The oligomeric states of haemagglutinin proteins were characterized by non-reducing SDS-PAGE. The bio-function of purified haemagglutinin proteins was then characterized by haemagglutination assay.

MATERIALS AND METHODS

Designing of transgenic vectors

Amino acid sequence of H5TG full-length haemagglutinin of A/duck/Vietnam/TG24-01/2005 influenza virus strain was codon-optimized and synthesized commercially in pUC57 vectors by GENECUST EUROPE company (in Luxembourg, Germany). The H5TG sequence encoding the ectodomain (aa 17–520) was amplified by PCR reaction using H5TG-BamHI-F and H5TG-PspOMI-R primers. Then H5TG was cloned into different three pRTRA vectors (being provided by IPK) in order to generate three expression cassettes with protein products of H5TG trimer, oligomer and ELP oligomer (as shown in Figure 1). In which, all of these expression cassettes contained a trimeric (GCN4-pII) domains in the C-terminus of H5TG (Harbury *et al*., 1993), a c-myc tag for detection H5TG proteins by Western blot, a His tag for purification of H5TGpII and H5TGpII-IgMFc proteins by IMAC and a legumin B4 signal peptide and the KDEL motif for promoting H5TG proteins retention in the endoplasmic reticulum. The H5TG proteins were under the control of CaMV 35S Pro promoter and CaMV 35S Term terminator. Subsequently, the expression cassettes of H5TG proteins in the pRTRA vectors were sub-cloned into the pCB301 vectors to generate three transgenic vectors (pCB301_H5TGpII; pCB301_H5TGpII-IgMFc and pCB301_H5TGpII-ELP-IgMFc). These vectors were transferred into *Agrobacterium tumefaciens* strain CV58 pGV2260 for transformation into plants.

Agroinfiltration

The expression of H5TG proteins in *Nicotiana benthamiana* plant by agroinfiltration assay was described in detail by Phan and Conrad (2016). *Agrobacterium tumefaciens* harboring the shuttle vectors for the expression of H5TG and HcPro proteins were pre-cultivated singly in LB medium containing 50 μg/mL kanamycin, 50 μg/mL carbenicillin and 50 μg/mL rifampicin at 140

rpm, 28°C and overnight. The pre-cultures were next added some more new LB culture containing the appropriate antibiotics. After 24 h of cultivation, the bacteria were harvested by centrifugation (5000 rpm, 30 min, 4°C) and resuspended in infiltration buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM $MgSO_4$, pH 5.6). These bacteria were combined and diluted in infiltration buffer until a final OD_{600} of 0.8-1.0. *N. benthamiana* plants (5 weeks old) were infiltrated using bacteria suspension solution by vacuum. Then, the plants were transferred into the greenhouse at 21°C and 16 h light per day. Post five days infiltration, tobacco leaf samples were harvested and stored at −80°C until use.

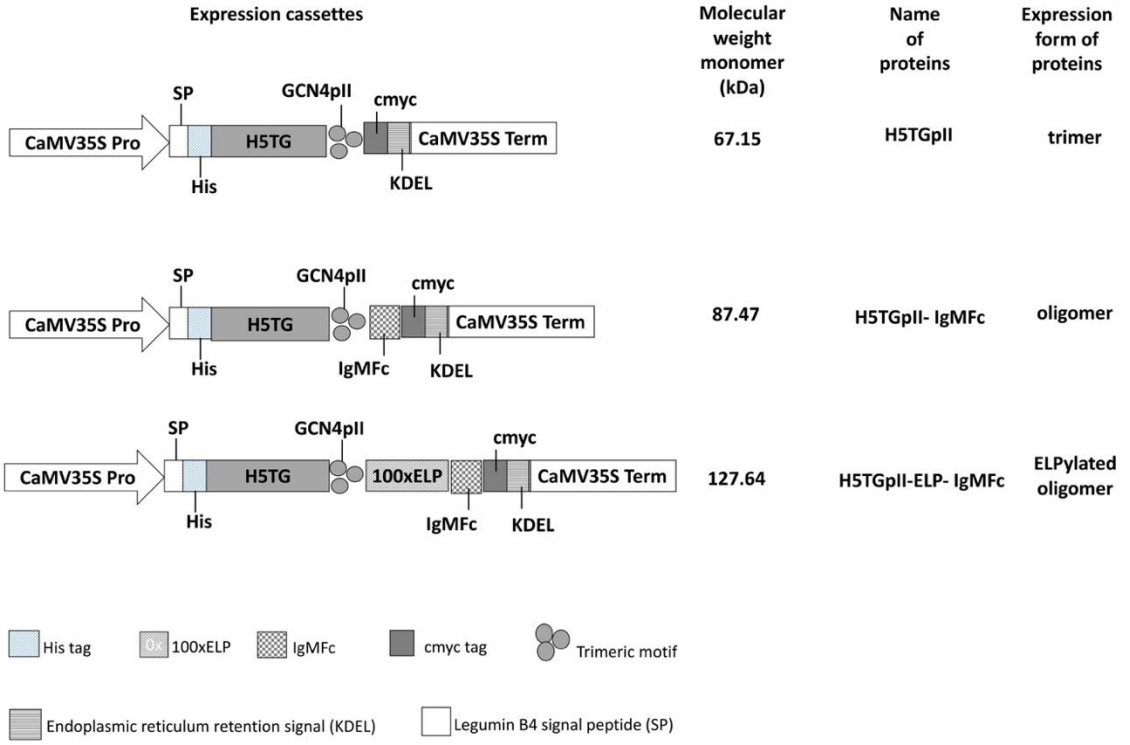

Figure 1. Expression cassettes of the H5TG proteins in plants. The ectodomain of haemagglutinin (HA) was trimerized by c-terminal fusion of trimeric motif (GCN4pII). The H5TG proteins were fused to a c-myc tag for detection by Western blot, and to a His tag for purification by IMAC. The legumine B4 signal peptide and the KDEL motif were used to promote transgene products retention in the endoplasmic reticulum. CaMV35S Pro: caulifower mosaic virus 35S ubiquitous promoter; CaMV 35S Term: caulifower mosaic virus 35S terminator. The IgM-Fc sequence (code P01872) of IgM from mice is optimized for the expression code in tobacco plants, synthesized by Geneart GmBh company.

Purification of H5TGpII and H5TGpII-IgMFc protein using immobilized metal ion affinity chromatography (IMAC)

The IMAC was based on the procedure of Phan and colleagues (2017) with some modifications. 40 g of leaf samples were ground in liquid nitrogen and homogenized in 120 mL of 50 mM Tris buffer (pH 8.0) using a commercial blender. The extracts were then clarified by centrifugation (10000 rpm, 30 min, 4°C). The supernatant solution was transferred into a new centrifugation tube and repeated centrifugation with the same condition. The clarified extracts

were then mixed with Ni–NTA agarose resin. After mixing for 1 h at 4°C, the mixture was added to a chromatography column. Thereafter, the column was washed by a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM of imidazole, pH 8.0). Recombinant proteins were then eluted from the column with 10 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 8.0). The proteins were put into dialysis bags for concentrating in PEG 6000 and subsequently dialyzed in a non-NaCl PBS buffer (2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The IMAC-purified H5TG proteins were kept at 50% of glycerol and stored at -20°C until use.

Purification of H5TGpII-ELP-IgMFc protein using membrane-based Inverse Transition Cycling (mITC)

The mITC was based on the procedure of Phan and Conrad (2016) with some modifications here. 50 g of the transiently transformed *N. benthamiana* leaves containing recombinant H5TGpII-ELP-IgMFc protein were ground in liquid nitrogen and the resulting powder was homogenized in 80 mL ice-cold 50 mM Tris-HCl (pH 8.0). The plant extract was cleared by centrifugation three times (10000 rpm, 45 min, 4°C) before the addition of a series of PEG 8000 from 0, 8, 10, 12, 14 and 20% (w/v). Then the cold extract was added NaCl to a final concentration of 2 M. The cold extract with 2 M NaCl was centrifuged again at 10000 rpm for 45 min at 4°C. The extract with 2 M NaCl was passed through a 0.3 µm mixed cellulose ester membrane and then through a 0.22 µm polyethersulfone membrane with the temperature maintained at 4°C. This filtrate was centrifuged again at 10000 rpm, 30 min, 4°C to produce a pre-treated extract. The pre-treated extract was warmed to room temperature and passed through a 0.2 µm cellulose acetate membrane using a vacuum pump. The membrane was washed twice with 2 M NaCl to remove non ELPylated contaminating proteins. Ice-cold Millipore-Q water was then passed through the filter to elute the protein-ELP fusions.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were following the protocol described by Phan and Conrad (2016) and were described briefly here. Extracted plant proteins, purified H5TG proteins in 2xSDS sample buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 0.2% (w/v), bromophenol blue, 20% (v/v) glycerol, pH 8.3) were separated by reducing SDS-PAGE (10% polyacrylamide) and then electrotransferred to nitrocellulose membranes at 18 V, overnight. After blocking for 2 h in TBS buffer (20 mM Tris–HCl, 180 mM NaCl) containing 5% (w/v) fat-free milk powder, the membranes were incubated at room temperature for 2 h with the addition of a 1:50 dilution of monoclonal anti-c-myc antibody. Next, the membranes were incubated with a 1∶2000 dilution of HRP conjugated goat anti-mouse IgG secondary antibody. The membranes were washed three times with TBS buffer containing 0.5% w/v fat-free milk and final wash times in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Finally, the signal of H5TG proteins was detected by using enhanced chemiluminescence (ECL) kit.

Haemagglutination test

The haemagglutination test was based on a standard protocol (OIE, 2004). The dilution that induced complete haemagglutination was defined as one haemagglutination unit (HAU). A 50 µL aliquot of total solution protein or purified proteins was placed in the first well of a microtiter plate containing 50 µL PBS, and two-fold serial dilutions were made across the row of 12 wells. Then, 50 µL of 1% chicken red blood cells was added, and the plates were incubated at 25 °C for 30 min. The HA titer is presented as the reciprocal of the highest dilution of protein that could cause complete agglutination of the red blood cells.

RESULTS AND DISCUSSION

Design of expression cassettes

In order to express the desired recombinant

H5TG proteins in a plant, these genes needs to be inserted into the plant transgenic vector. In this study, the pCB301 transgenic vector was used to carry the expression structures of the desired target H5TG proteins (Figure 1). Firstly, three these expression cassettes of H5TGpII; H5TGpII-IgMFc and H5TGpII-ELP-IgMFc were cloned successfully in pRTRA vector. In particular, the H5TG segment was attached to the GCN4pII motif to create the trimeric H5TGpII expression cassettes that carries three haemagglutinin molecules similar to those in nature. The H5TGpII segment was fused with an IgMFc motif to form the oligomeric H5TGpII-IgMFc protein via the S-S binding in IgMFc. For designing of the ELPylated oligomeric H5TGpII-ELP-IgMFc protein, the H5TGpII was fused to an ELP-IgMFc combination containing a 100xELP fragment with the aim of enhancing the expression as well as facilitating purification of the H5TG protein more easily by membrane based Inverse Transition Cycling (mITC). These constructs also contained a c-myc tag for detection H5 proteins by Western blot, a His tag for purification of H5 proteins by IMAC and a legumin B4 signal peptide and the KDEL motif for promoting these proteins retention in the endoplasmic reticulum. The resulting three expression cassettes were then subcloned successfully into the pCB301 transgenic vector *via* restriction enzyme sites (Figure 2). The electrophoresis image analysis of cutting the pCB301_H5TGpII, pCB301_H5TGpII-IgMFc and pCB301_H5TGpII-ELP-IgMFc plasmids using *Nco*I restriction enzyme shows the obtained fragments as calculated.

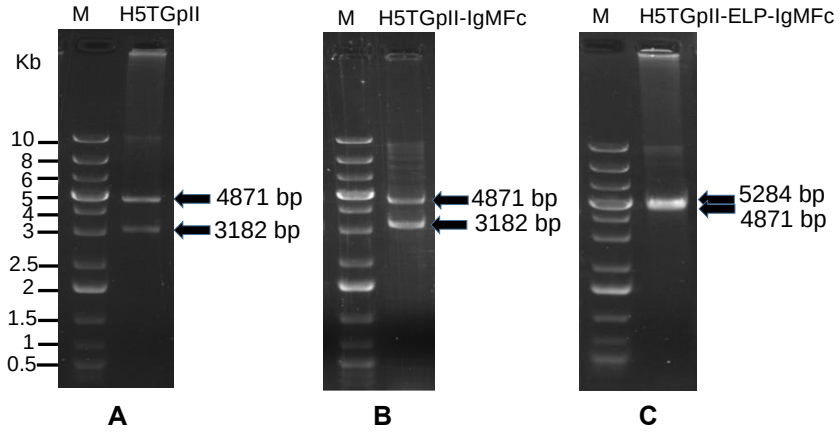

Figure 2. The electrophoresis image of the pCB301_H5TGpII (A), pCB301_H5TGpII-IgMFc (B) and pCB301_H5TGpII-ELP-IgMFc (C) plasmids with the opposite expression being expressed for the gene coding for the kanamycin-resistant npt protein on themself vectors were cut by *Nco*I restriction enzyme. M: GeneRuler 1 kb Plus DNA Ladder (Fermentas).

Expression and bio-functional characterization of H5TG proteins in plant raw extract

The transient expression in tobacco using agroinfiltration is an effective method to produce subunit vaccines from plants because of low production cost and infrastructure investment, easily scale up as well as high product stability (Chen *et al.*, 2013; Topp *et al.*, 2016). In this study, the proteins H5TGpII, H5TGpII-IgMFc and H5TGpII-ELP-IgMFc were transformed successfully into *N. benthamiana via* vacuum agroinfiltration analyzed by reducing SDS-PAGE and Western blot. These proteins have been expressed in tobacco leaves, in which the H5TGpII-ELP-IgMFc protein was stronger expressed than the remaining proteins (Figure 3).

A strong benefit of protein expression in plants is that the biological activity of the protein is maintained. Haemagglutinin assay is a technique to evaluate the ability of haemagglutinin to agglutinate to the red blood cells based on the association of sialic acid sites on chicken red blood cells. The results of haemagglutinin assay are shown in Figure 4. The HA titer of the H5TGpII-IgMFc protein (64) is higher than the HA titer of the H5TGpII (2) and H5TGpII-ELP-IgMFc (8) proteins. Equal amounts of total protein solution were applied. The H5TGpII-ELP-IgMFc protein showed the highest expression, and also the highest HA titer.

Purification of the H5TG proteins

In order to exactly describe and compare the bio-functions of H5TG proteins, raw extracts of each H5TG protein were used to apply different methods of purification. For purification of the H5TGpII and H5TGpII-IgMFc proteins, IMAC method was used. The results in the Figure 5 show that the purified proteins (as shown in Line 4) show very clear and clean bands, and losses by flow through and washing steps are negligible.

For purification of the H5TGpII-ELP-IgMFc protein, mITC was used based on the procedure of Phan and Conrad (2016) with some modifications, here. The mITC procedure was optimized to enrich ELPylated H5TGpII-ELP-IgMFc protein from transiently transformed leaf materials using PEG 8000. It can also be used for purification of ELPylated proteins from transgenic plant leaves to reduce high-speed centrifugation steps that were required to have enough clearance of plant extract for mITC as described above. Plant extracts from transiently transformed leaf materials are difficult to pass through a 0.22 µm polyethersulfone membrane at 4ºC even if they were clarified by high speed centrifugation. Therefore, in this study we used polyethylene glycol (PEG) to precipitate plant soluble contaminants that were then simply removed by low speed centrifugation (Figure 6). The optimal PEG 8000 concentration was 8-10% (w/v), because at these concentrations the target protein was still present in the supernatant as determined by Western blot and Coomassie staining (Figure 6b and c). Then, to determine clearly the enrichment of a majority of the target H5 protein of PEG 8000, NaCl was added to the supernatant (with and without PEG 8000 (9%)). The resulting solution was used for mITC procedure with four different ITC rounds. The target protein was eluted and detected by Coomassie staining as shown in Figure 7a and 7b. The results show that an enrichment of a majority of the target protein after the first round of mITC with PEG while it was distributed equally in every mITC round that did not use PEG. This result indicated that PEG 8000 enhanced dramatically the maintenance of ELPylated H5 on the membrane. For purification of large amount of tobacco extract containing the H5TGpII-ELP-IgMFc protein by mITC with PEG 8000 (9%), the H5TGpII-ELP-IgMFc protein was almost completely obtained (as shown in Figure 7c and 7d).

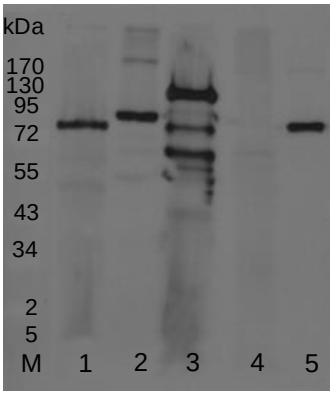

Figure 3. The transient expression of H5TGpII (Line 1), H5TGpII-IgMFc (Line 2) and H5TGpII-ELP-IgMFc (Line 3) in tobacco raw extract. 30 µg TSP of raw extract were separated by reducing SDS-PAGE and detected by Western blot using anti-cmyc antibody and ECL analysis; Line M, Marker of reference proteins; Line 4: raw extract of wild type tobacco as a negative control; Line 5: 50 ng of purified Haemagglutinin protein using as a positive control.

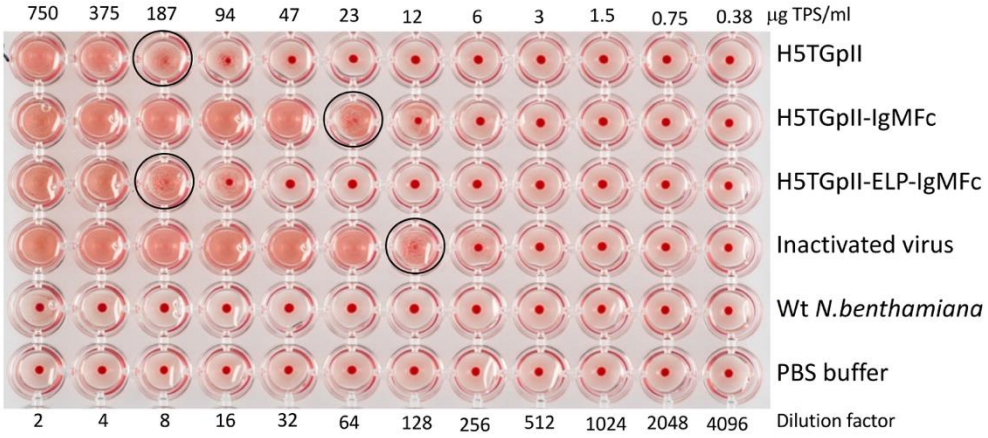

Figure 4. Haemagglutination assay of the total proteins containing H5TG. 1500 µg (~50 µL) of total protein extract per structure and non-transgenic plants were added to the first well on the bottom plate V. 50 µL of PBS buffer was used for negative control. Inactivated A/H5N1 virus is used as positive control for erythrocyte agglutination reaction. After that, a series of two dilutions is transferred to the next well until the last well. 50 µL of 1% chicken red blood cells was added to all wells. Results are read after 30 minutes of incubation at 25°C.

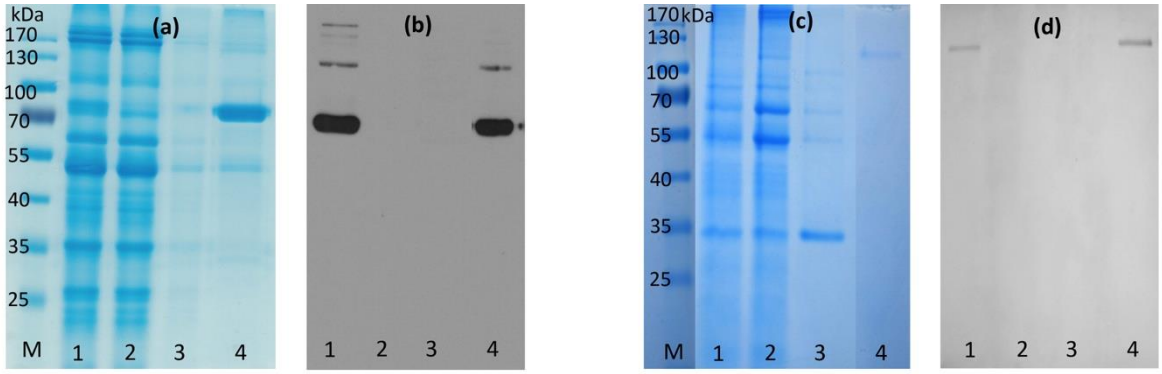

Figure 5. Purification of the H5TGpII protein (a, b) and H5TGpII-IgMFc protein (c, d) by IMAC. Proteins were separated via SDS-PAGE at reducing conditions using a 10% polyacrylamide gel, and then detected by SDS-PAGE staining with Coomassie Brilliant blue R-250 reagent (a, c) and visualized by Western blot (b, d). Line M: Marker of reference proteins; Line 1, 2 and 3 are 15 µL (a and b) and 30 µg (c and d) total solution protein (TSP) of raw extract, flow through and wash fraction, respectively; Line 4a: 50 µL of the purified H5TGpII protein; Line 4b: 1,75 µL of the purified H5TGpII protein; Line 4c: 2 µg of the purified H5TGpII-IgMFc protein; Line 4d: 100 ng of the purified H5TGpII-IgMFc protein.

Structural and bio-functional characterization of the purified H5TG proteins

By SDS-PAGE at non reducing conditions, the oligomeric structure of purified H5TG proteins was analyzed *via* SDS-PAGE at non reducing conditions and Western blotting based on an anti-c-myc monoclonal antibody (Figure 8a). In particular, the size of the H5TGpII proteins was about 200 kDa (Line 2), while the H5TGpII-IgMFc and H5TGpII-ELP-IgMFc proteins were detected above the 210 kDa (Line 1) and above the 260 kDa (Line 3), respectively. Purified H5TG proteins were also analyzed by Haemagglutination assay as shown in Figure 8b. Only 0.06 µg of the purified H5TGpII-IgMFc protein can agglutinate red blood cells, while at least 0.24 µg of purified H5TGpII or purified H5TGpII-ELP-IgMFc proteins were needed for agglutination.

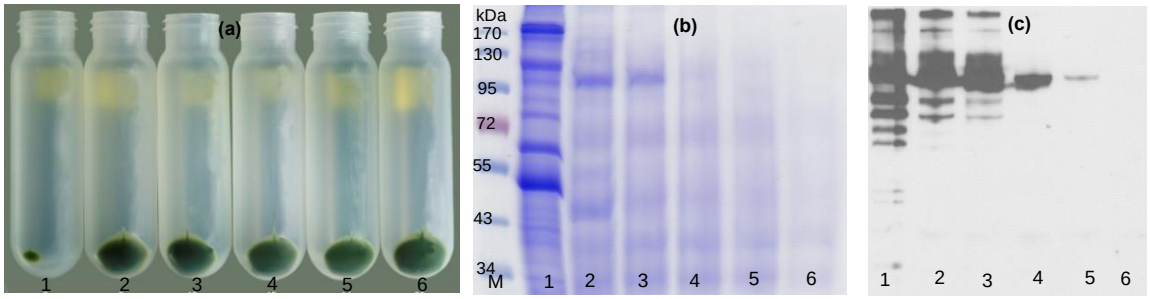

Figure 6. Removal of plant soluble contaminants by different concentrations of PEG 8000. Plant proteins containing the H5TGpII-ELP-IgMFc recombinant protein were extracted in Tris buffer, pH 8.0. Centrifugation was applied to remove plant cell debris. PEG 8000 was added into the clear plant extract to precipitate plant soluble contaminants. The resulting solution was clarified by centrifugation. Proteins in supernatants were then used for electrophoresis and detected by Coomassie blue (b) and Western blot (c), while pellets were settled in the bottoms (a). Line 1-6: a series of 0, 8, 10, 12, 14 and 20% concentration of PEG 8000 (w/v).

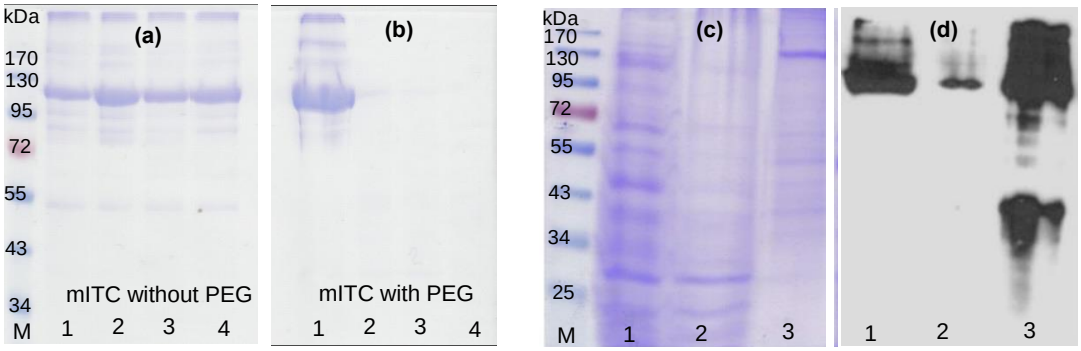

Figure 7. Purification of the H5TGpII-ELP-IgMFc protein by mITC with PEG 8000 (9%). Proteins were separated via SDS-PAGE at reducing conditions using a 10% polyacrylamide gel, and were then detected by staining with Coomassie Brilliant blue R-250 reagent (a, b, c) and visualized by Western blotting based on an anti-c-myc monoclonal antibody (d). Line M: Marker of reference proteins; Line 1-4a and b, plant extract containing sodium chloride (2M) with and without PEG 8000 (9%), respectively, were used for mITC with four rounds, equal volumes (40 µL) of eluted proteins were loaded in each line; Line 1-3c and d, plant extract containing sodium chloride (2M) with PEG 8000 (9%); Line 1c and d, proteins in the raw plant extract; Line 2c and d, proteins in the supernatant after passage through a 0.2 µm cellulose acetate membrane; Line 3 c and d, proteins in the elution.

Many studies demonstrated that oligomeric and trimeric haemagglutinins could induce efficiently neutralizing antibodies in vaccinated animal (Bosch *et al*., 2010; Weldon *et al*., 2010; Phan *et al*., 2013; Phan *et al*., 2017). As higher the haemagglutination titre of HA, as higher is the ability to stimulate the production of neutralizing antibodies. Stimulation of the production of highly neutralizing antibodies of HA in vaccinated animals is an extremely important factor, determining whether that HA antigen is likely to be used as a vaccine to protect poultry or not. The capacity to stimulate neutralizing antibodies can be measured by the haemagglutination inhibition assay. So, scientists are always looking for ways to design recombinant HA structures with a higher ability to agglutinate red blood cells. In 2013, Phan and co-workers studied one recombinant HA trimer fusing ELP (named as ^{Nb}(H5pII-ELP)3) was able

to agglutinate red blood cell while HA monomer (named as ^{Nt}H5-ELP) was not. And the result in HI titres against homologous VLPs measured in sera of vaccinated mice of ^{Nb}(H5pII-ELP)3 was higher 4.2 fold than ^{Nt}H5-ELP and ELP fusions enhanced accumulation of recombinant HA proteins in tobacco, so the purification of HAs were easier (Phan *et al*., 2013). In 2017, Phan and co-workers showed that the HA titer of HA oligomer (HAU=256) was higher 32 fold than HA trimer (HAU=8), and H5 oligomer (HI titre = 53.8) stimulated neutralizing antibody production in mice was higher 5 fold than H5 trimer (HI titre = 10.7) (Phan *et al*., 2017). In this study, the similar result of ELP fusion enhanced more accumulation of the recombinant H5TGpII-ELP-IgMFc protein in *N. benthamiana* than accumulation of the H5TGpII and H5TGpII-IgMFc proteins. However, H5TGpII-IgMFc protein, having the strongest and highest HA titre, will be a potential vaccine candidate with the high ability of inducing efficiently neutralizing antibodies against A/H5N1. These results showed that the using of GCN4pII-IgMFc motif to form oligomer HA protein enhanced bio-function of that protein.

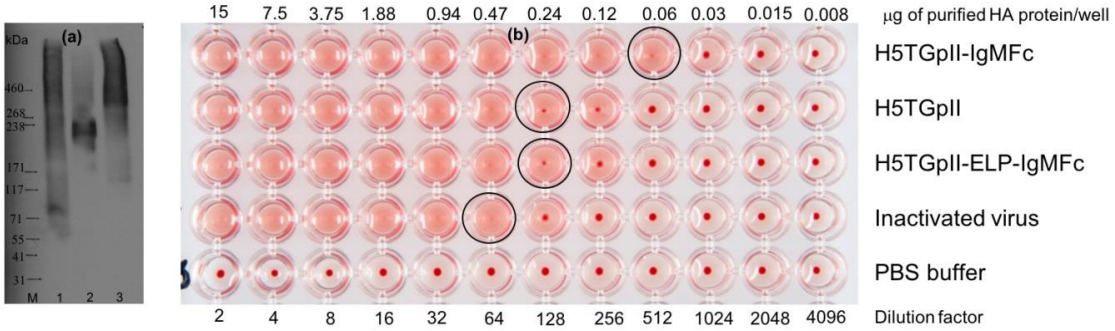

Figure 8. Structural characterization of purified H5TG proteins by SDS-PAGE at non reducing conditions using a 10% polyacrylamide gel and visualized by Western blotting based on an anti-c-myc monoclonal antibody (a) and bio-functional characterization of the purified H5TG proteins by Haemagglutination assay (b). Line M: Marker of reference proteins; Line 1a, H5TGpII-IgMFc; Line 2a, H5TGpII; Line 3a, H5TGpII-ELP-IgMFc. PBS buffer was used for negative control. Inactivated A/H5N1 virus is used as positive control for erythrocyte agglutination reaction.

CONCLUSION

The trimeric H5TGpII, oligomeric H5TGpII-IgMFc and ELPylated oligomeric H5TGpII-ELP-IgMFc proteins have been generated and successfully expressed in *N. benthamiana* by agroinfiltration. The oligomeric H5TGpII-IgMFc protein had the strongest biofunctional activity. This result opens the way to apply the GCN4pII-IgMFc motif for generation of oligomeric proteins to enhance the biological activity of target proteins in studies of recombinant vaccine production.

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SỰ TĂNG CƯỜNG CHỨC NĂNG SINH HỌC CỦA HAEMAGGLUTININ Ở THỰC VẬT DỰA VÀO SỰ DUNG HỢP VỚI IgMFc

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TÓM TẮT

Việc nghiên cứu tạo protein haemagglutinin tái tổ hợp của virus A/H5N1 dạng oligomer là vấn đề mới được nhiều nhà khoa học quan tâm. Trong nghiên cứu này, gen mã hoá protein haemagglutinin (H5TG) có nguồn gốc từ virus A/duck/Vietnam/TG24-01/2005 được dung hợp với ba loại motif khác nhau (GCN4pII, GCN4pII-IgMFc và GCN4pII-ELP-IgMFc) để lần lượt tạo ra ba loại protein tái tổ hợp (H5TGpII dạng trimer, H5TGpII-IgMFc dạng oligomer và H5TGpII-ELP-IgMFc dạng oligomer ELP hoá) nhằm tăng cường mức độ biểu hiện, hoạt tính sinh học và khả năng tinh sạch của protein H5TG. Các đoạn gen H5TG này đã được gắn vào cassette biểu hiện trong vector chuyển gen pCB301 và được biểu hiện tạm thời thành công trên thuốc lá *Nicotiana benthamiana* bằng phương pháp agroinfiltration thông qua phân tích SDS-PAGE và Western blot. Kết quả phân tích cho thấy, protein H5TGpII-ELP-IgMFc có sự biểu hiện trong thuốc lá cao nhất so với hai protein tái tổ hợp còn lại. Đánh giá hoạt tính sinh học bằng phản ứng ngưng kết hồng cầu nhận thấy, hiệu giá ngưng kết hồng cầu của dịch chiết protein tổng số chứa protein H5TGpII-IgMFc đạt cao nhất (64 HAU), trong khi hiệu giá ngưng kết hồng cầu của dịch chiết protein tổng số chứa hai protein còn lại chỉ đạt 8 HAU. Các protein H5TG sau khi được tinh sạch bằng IMAC (đối với protein H5TGpII và H5TGpII-IgMFc) và tinh sạch qua màng mITC (đối với protein H5TGpII-ELP-IgMFc) tiếp tục được xác định trạng thái

oligomer thông qua SDS-PAGE không biến tính và đánh giá hoạt tính sinh học bằng phản ứng ngưng kết hồng cầu. Phân tích đã cho thấy, hàm lượng protein nhỏ nhất để gây ngưng kết hoàn toàn tế bào hồng cầu gà (1 HAU) của protein H5TGpII-IgMFc là 0,06 µg ít hơn bốn lần so với 1HAU của hai protein H5TGpII và H5TGpII-ELP-IgMFc (cả hai đều là 0,24 µg). Điều này chứng tỏ rằng việc dung hợp motif GCN4pII-IgMFc vào protein H5TG đã cho hoạt tính sinh học cao hơn so với hai motif còn lại. Kết quả này đã mở ra khả năng ứng dụng IgMFc trong việc tạo oligomer khi dung hợp IgMFc với các protein khác nhằm tăng cường hoạt tính sinh học của protein đích trong nghiên cứu tạo vaccine tái tổ hợp.

Từ khoá: *haemagglutinin, IMAC, mITC, oligomer, phản ứng ngưng kết hồng cầu.*