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Immunological study of HA1 domain of hemagglutinin of influenza H5N1 virus

Fang-Feng Chiu [a], Nandini Venkatesan [a], Chia-Rong Wu [a], Ai-Hsiang Chou [a], Hsin-Wei Chen [a], Shu-Pei Lian [a], Shih-Jen Liu [a], Chin-Cheng Huang [c], Wei-Cheng Lian [a,b], Pele Chong [a], Chih-Hsiang Leng [a,*]

[a] Vaccine Research and Development Center, National Health Research Institutes, 35 Keyan Road, Zhunan, Miaoli 350, Taiwan, ROC
[b] Center for Disease Control, Taipei 105, Taiwan, ROC
[c] Animal Health Research Institute, Council of Agriculture, 376 Chung-Cheng Road, Tamsui, Taipei 251, Taiwan, ROC

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ABSTRACT

The neutralization titer of a hemagglutinin (HA)-specific neutralizing antibody against new isolates reflect both the antigenic drift and the conformation status of HA protein in these new influenza viruses. Since most antigenic sites are in the HA1 domain of HA, using HA1 domain of influenza virus as antigen is of great importance in vaccine development. In this study, we investigate different purification processes for optimizing the immunological properties of an *Escherichia coli*-expressed HA1 domain (rH5HA1) of influenza H5N1 virus. rH5HA1 was expressed as inclusion bodies and extracted with 6 M guanidine hydrochloride (GnHCl)/PBS buffer. The best condition for generating HA1-specific neutralization determinants is on-column oxidative refolding procedures with GSH/GSSG and L-arginine buffer. Others refolding procedures such as using high-pH buffer and/or different detergent solubilizations were found to be ineffective producing neutralization epitope recognized by a HA1-specific neutralizing monoclonal antibody that was raised against H5N1 virus.

Introduction

Influenza is a highly contagious, acute, viral respiratory disease which causes significant morbidity and mortality worldwide each year [1,2]. Global outbreaks of human influenza (pandemics) arise from influenza A viruses with novel hemagglutinin (HA) and/or neuraminidase (NA) molecules to which humans have no immunity [3]. Specifically, the structural changes and antigenic variations in the HA molecule are the main obstacle to the control of viral transmission [4]. The HA1 domain of HA almost contains all of the antigenic sites, and is more prone to mutations and antigenic drifts than the rest of protein [5]. Previous studies have demonstrated that the antigenic sites are conformational epitopes [6,7]. Consequently, structural and antigenic variations in the HA1 domain can be reflected in its binding to neutralizing monoclonal antibodies. In addition to containing these conformational epitopes, HA1 also plays an important role in the refolding of HA to its fusion competent conformation [8,9]. Thus, obtaining a HA1 subunit in a correctly-folded state is imperative to eliciting the correct antibody response; this is a crucial part of drug and vaccine development [10,11].

Since *Escherichia coli* system offers a means for the rapid and economical production of recombinant proteins, we wished to use it for the production of recombinant HA1. More importantly, we focused on producing this antigen in a properly folded conformation and tested whether the recombinant HA1 could be refolded without post-translational modification. Using an on-column refolding procedure, we successfully purified and refolded the recombinant HA1 of the H5 hemagglutinin (rH5HA1) from inclusion bodies. Simultaneously, rH5HA1 preparations were purified using a high-pH buffer or detergent. The rH5HA1 prepared by the latter two methods and overlapping peptides spanning the entire sequence of the rH5HA1 were assayed to determine whether they could be recognized by a neutralizing monoclonal antibody. Our results demonstrate that oxidatively-refolded rH5HA1 is likely correctly folded.

Materials and methods

Cloning of the recombinant HA1 protein. The consensus sequence of HA1 (H5HA1) from the HA of influenza virus was synthesized using assembly PCR method [12]. The H5HA1 gene was PCR-amplified using the forward primer 5′GGAATTCCATATGGAGAAGAT CGTGCTG and the reverse primer 5′CCGCTCGAGACGCTTCTTAGA CGACGCT to introduce Nde1 and Xho1 sites (underlined). The PCR product was cloned into the expression vector, pET-22b (+) (Novagen, Madison, USA), resulting in the plasmid pH5HA1, which encoded the protein with a C-terminal His-tag.

Purification of rH5HA1 protein from inclusion bodies. After expression of rH5HA1, cells harvested from 3.6 L of culture were disrupted with a French Press (Constant Systems, Daventry, UK) at

* Corresponding author. Fax: +886 37 583 009.
E-mail address: leoleng@nhri.org.tw (C.-H. Leng).

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27 Kpsi and clarified by centrifugation (80,000*g* for 60 min). The induced protein was extracted from the cell pellet using 90 ml of 6 M guanidine hydrochloride (GnHCl)/phosphate-buffered saline (PBS)/10 mM imidazole (pH 7.6). The "non-native" rH5HA1 was purified using an IMAC column (Ni^{2+}–NTA resin). The relative protein concentration was determined by the Bradford method [13] and the bicinchoninic acid (BCA) assay, using bovine serum albumin (BSA) as a standard.

Preparing soluble forms of the denatured protein by three different methods. Three methods were used to prepare the soluble form of rH5HA1 free of chaotropic agents. First, the on-column folding procedure from Guo et al., 2006 [14] was used with some modifications. Briefly, seven milligrams of the GnHCl-extracted protein was applied to the column and the column was washed with 10 mL of binding buffer (6 M GnHCl, 10 mM imidazole in PBS, pH 7.6). The bound protein was folded on the column using 40 mL of folding buffer (50 mM imidazole, 1 mM GSSG/GSH and 400 mM L-arginine in PBS, pH 7.6). The protein was eluted with 5 mL of folding buffer with 500 mM imidazole and was dialyzed against dialysis buffer (PBS/1 mM GSSG/GSH/400 mM L-arginine, pH 7.6) for further studies. The protein obtained in this way was denoted as rH5HA1o. The second method employed a detergent-based buffer containing 2% Tween-20 in PBS with 500 mM imidazole (pH 7.6). The protein was eluted from IMAC using this buffer and was designated as rH5HA1d. In the last preparation, rH5HA1 was eluted in PBS with 500 mM imidazole (pH 7.6) and dialyzed against high-pH buffer containing 25 mM phosphate/20 mM β-mercaptoethanol (pH 11.9). A clear solution of rH5HA1 was obtained and was denoted as rH5HA1h.

Neutralization assay. Undiluted supernatants were incubated with 200 TCID50 (50% tissue culture infective doses) of virus for 1 h at room temperature prior to the addition to Madin-darby canine kidney (MDCK) cells. Determination of endpoint neutralizing antibody titers was performed. Briefly, samples were serially diluted two-fold, from a starting dilution of 1:40, prior to mixing with virus; residual infectivity was tested in four wells per dilution. The neutralizing titer was defined as the reciprocal of the highest dilution of serum at which the infectivity of 200 TCID50 of the H5N1 virus for MDCK cells was completely neutralized in 50% of the wells. Infectivity was identified by the presence of cytopathic effect on day 4 and the titer was calculated by the Reed–Muench method [15].

Binding assays of rH5HA1 preparations and H5HA1 derived synthetic peptides by ELISA. Two different sets of ELISA experiments were carried out. First, anti-rH5HA1h anti-sera were used for the binding assay, which provided information regarding the linear epitopes of H5HA1. The coated samples included a peptide "cocktail" of H5 covering H5HA1 sequences (Table 1), rH5HA1h, rH5HA1d, and rH5HA1o. The inactivated H5N1 virus was used as a positive control. The samples were incubated with the rabbit polyclonal antiserum or pre-immune serum made against rH5HA1h for 2 h. They were washed six times with PBST and incubated with HRP-conjugated goat anti-rabbit IgG as the secondary antibody at room temperature for 30 min. The wells were thoroughly washed eight times with PBST and the substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) was added (100 μL per well) and incubated at room temperature for 30 min. Subsequently, the reaction was stopped by adding 1 N H_2SO_4 and the binding was quantified by measuring the color development at an absorbance of 450 nm.

For the second set of ELISA experiments, Y5 monoclonal antibody was used to investigate the folding status of coating proteins/peptides. The samples were coated as described above. The primary antibody used was the Y5 monoclonal antibody to H5 (Kinmen strain) [16]; BALB/c serum was used to monitor the background absorbance. The binding was quantified as described above.

Antibody dilution and competition assay. The competition assay was performed by coating wells with inactivated NIBRG-14. In this assay, the Y5 antibodies were diluted at 1/25,000 and incubated with different amounts of samples before reaction with inactivated virus. Binding was measured as described above.

Determination of structural information using light scattering spectrometry. Dynamic light scattering (DLS) measurements were carried out on a DynaPro 99 MS800 instrument (Protein Solutions, Lakewood, NJ, USA) at 25 °C. The rH5HA1o (1 mg/ml) in a buffer of PBS/1 mM GSSG/GSH/400 mM L-arginine (pH 7.6), rH5HA1d (1 mg/ml) in a buffer of 2% Tween-20 in PBS with 500 mM Imidazole (pH 7.6), and rH5HA1h (1 mg/ml) in a buffer of 25 mM phosphate/20 mM β-mercaptoethanol (pH 11.9) were placed in a 12-μL cuvette ($b = 1.5$ nm). The protein samples were incubated for 5 min in 25 °C before data acquisition over an acquired time of 15 min. The size distribution plots, with the *x* axis showing a distribution of estimated particle radius (nm) and the *y* axis showing the relative intensity of the scattered light (% of mass), were analyzed and prepared with the software Dynamics V6.3.01 (Protein Solutions). Individual data of rH5HA1 preparations were combined and plotted using Microsoft Office Excel.

Results

Cloning, expression, and purification of the rH5HA1 expressed in E. coli

The sequences of HA1 domain of HA in three strains of H5 viruses, A/Vietnam/1194/2004 (H5N1), A/Hong Kong/483/97 (H5N1) and A/Thailand/LFPN-2004/2004 (H5N1) were aligned and a consensus sequence was obtained (Fig. 1A). This sequence was taken as representative of the HA1 gene of H5 hemagglutinin (H5HA1). The H5HA1 gene was amplified and cloned into the pET22b vector (Fig. 1B). The recombinant protein containing an additional six histidine residues (His-tag) at its C-terminal end was expressed under the control of a T7 promoter. The cloned plasmid, pH5HA1, was transformed into BL21 (DE3) strain and induced using 1 mM IPTG at 37 °C. The fraction containing the rH5N1 was isolated after solubilizing the inclusion body with 6 M GnHCl. Subsequently, "non-native" rH5HA1 was purified on an IMAC column in the presence of 6 M GnHCl (Fig. 1C, lanes 1–4). The purified rH5HA1 could be recognized by anti-His-tag antibodies (Fig. 1D, lanes 1–4). Since the purified "non-native" rH5HA1 precipitated during dialysis against PBS, different methods were used to obtain soluble preparations of rH5HA1.

Preparation of soluble rH5HA1s by different methods

With the aim of producing a correctly-folded form of the HA1 domain, three different methods was used to produce soluble preparations of rH5HA1 (see Materials and methods). Firstly, oxidative folding was carried out using a redox-shuffling buffer contained L-arginine in an IMAC column to produce rH5HA1o with a yield of 4.2 mg/L of culture. The other two methods employed were dialysis against a high-pH buffer (pH 11.9) to obtain rH5HA1h with a yield of 1.1 mg/L of culture, and elution from the IMAC column in a detergent-based buffer to obtain rH5HA1d with a yield of 2 mg/L of culture. These preparations of rH5HA1s (rH5HA1o/h/d) were digested by trypsin and characterized by MALDI-TOF spectrometry. The results confirmed that the major peaks in the mass spectra data were derived from rH5HA1 (data not shown). These preparations (rH5HA1o/h/d) were subsequently tested for their folding status via their ability to be recognized by the Y5 neutralizing monoclonal antibody.

Table 1
Amino acid sequences of the H5HA1 peptides and the HA-A to HA-E 'cocktails' representing linear epitopes of H5HA1.

Names	Peptide names	Sequence	Names	Peptide names	Sequence
HA-A	H5-01	MEKIVLLLAIVSLVK	HA-C	H5-26	IKRSYNNTNQEDLLV
	H5-02	LAIVSLVKSDQICIG		H5-27	TNQEDLLVLWGIHHP
	H5-03	KSDQICIGYHANNST		H5-28	VLWGIHHPNDAAEQT
	H5-04	GYHANNSTEQVDTIM		H5-29	PNDAAEQTKLYQNPT
	H5-05	TEQVDTIMEKNVTVT		H5-30	TKLYQNPTTYISVGT
	H5-06	MEKNVTVTHAQDILE	HA-D	H5-31	TTYISVGTSTLNQRL
	H5-07	THAQDILEKTHNGKL		H5-32	TSTLNQRLVPEIATR
	H5-08	EKTHNGKLCDLDGVK		H5-33	LVPEIATRSKVNGQS
	H5-09	LCDLDGVKPLILRDC		H5-34	RSKVNGQSGRMEFFW
	H5-10	KPLILRDCSVAGWLL		H5-35	SGRMEFFWTILKPND
HA-B	H5-11	CSVAGWLLGNPMCDE		H5-36	WTILKPNDAINFESN
	H5-12	LGNPMCDEFINVPEW		H5-37	DAINFESNGNFIAPE
	H5-13	EFINVPEWSYIVEKA		H5-38	NGNFIAPEYAYKIVK
	H5-14	WSYIVEKANPVNDLC		H5-39	EYAYKIVKKGDSTIM
	H5-15	ANPVNDLCYPGDFND		H5-40	KKGDSTIMKSELEYG
	H5-16	CYPGDFNDYEELKHL	HA-E	H5-41	MKSELEYGNCNTKCQ
	H5-17	DYEELKHLLSRINHF		H5-42	GNCNTKCQTPMGAIN
	H5-18	LLSRINHFEKIQIIP		H5-43	QTPMGAINSSMPFHN
	H5-19	FEKIQIIPKSSWSSH		H5-44	NSSMPFHNIHPLTIG
	H5-20	PKSSWSSHEASLGVS		H5-45	NIHPLTIGECPKYVK
HA-C	H5-21	HEASLGVSSACPYQG		H5-46	GECPKYVKSNRLVLA
	H5-22	SSACPYQGKSSFFRN		H5-47	KSNRLVLATGLRNSP
	H5-23	GKSSFFRNVVWLIKK		H5-48	ATGLRNSPQRERRRK
	H5-24	NVVWLIKKNSTYPTI		H5-49	PQRERRRKKR
	H5-25	KNSTYPTIKRSYNNT			

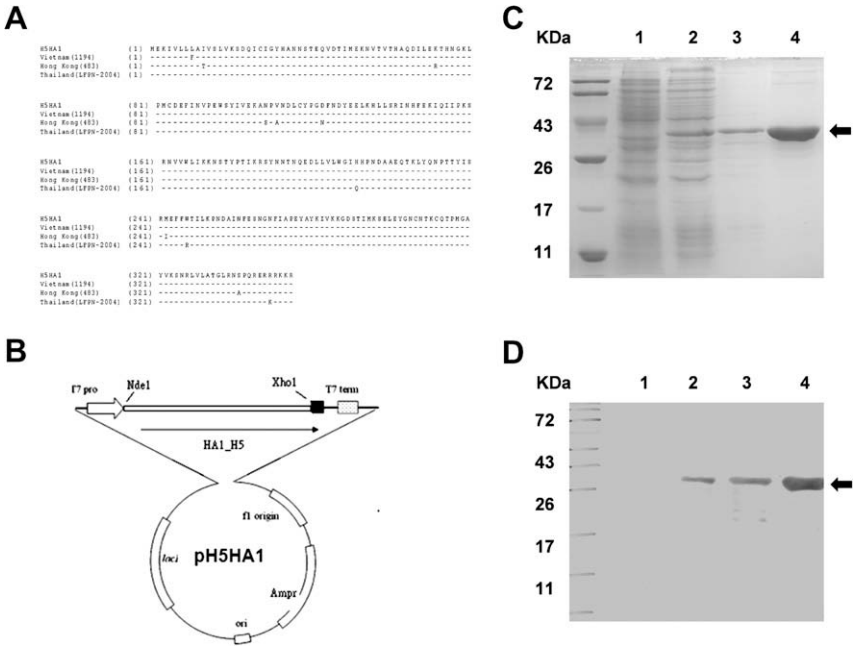

Fig. 1. Construction, expression, and purification of rH5HA1 in *E. coli* BL21(DE3) cells. (A) The sequences of HA1 domain of HA in three strains of H5 viruses, A/Vietnam/1194/2004 (H5N1), A/Hong Kong/483/97 (H5N1), and A/Thailand/LFPN-2004/2004 (H5N1) were aligned and a consensus sequence, H5HA1, was obtained. (B) The H5HA1 gene was synthesized and cloned into the pET22b vector. The recombinant protein contained an additional HHHHHH sequence (His-tag) at its C-terminal end and could be expressed under the control of T7 promoter. (C) The "non-native" rH5HA1 was purified using IMAC column in the present of 6 M GnHCl. Lane 1, cell lysate before IPTG induction; lane 2, cell lysate after IPTG induction; lane 3, fraction of induced cells extracted with 6 M GnHCL; lane 4, the purified "non-native" rH5HA1. The arrow indicates the position of "non-native" rH5HA1 in the SDS–PAGE. (D) The immunoblot monitoring of "non-native" rH5HA1 purification process, using anti-(His)6 antibodies. The arrow indicates the position of "non-native" rH5HA1 in the blot.

NIBRG-14 virus can be neutralized by Kinmen strain-derived neutralizing monoclonal antibody

The Y5 neutralizing monoclonal antibody was derived from the Kinmen strain of the H5N1 virus [16]. Since we wished to perform our neutralizing assay using NIBRG-14 virus (engineered by Dr. John Wood at NIBSC), we first tested to see whether the NIBRG-14 virus could also be neutralized by the Y5 antibody. Table 2 shows the results of the neutralization assay of the Y5 monoclonal antibody. The anti-sera generated from inactivated NIBRG-14 virus and rNtM2-NP protein are included for comparison. The results showed that the NIBRG-14 virus is neutralized by the Y5 monoclo-

Table 2
The neutralization titer of NIBRG-14 virus by the Y5 monoclonal antibody, anti-sera generated from rNtM2-NP, and anti-sera generated from inactivated NIBRG-14 virus.

	Y5 monoclonal antibody	Anti-sera generated from rNtM2-NP	Anti-sera generated from inactivated NIBRG-14 virus
$TCID_{50}$ titer	753.5	25.24	1422.6

nal antibody. Therefore, the binding of Y5 monoclonal antibody to the rH5HA1 preparations could reflect the presence of antigenic sites and the structural integrity of rH5HA1 preparations.

Oxidatively-folded rH5HA1 can compete with inactivated virus for the binding of Y5 antibody

Since the binding of Y5 monoclonal antibodies depends on the folding of HA1, the folding status of different preparations of rH5HA1 (rH5HA1o/h/d) could be determined by ELISA using selected antibodies. First, several samples including five groups of peptides representing linear epitopes (Table 1), rH5HA1o/h/d and the inactivated virus were coated on plates. These proteins were detected by rabbit anti-rH5HA1h antibodies (see Materials and methods) which cannot neutralize the NIBRG-14 virus (data not shown). The results showed that the rH5HA1o/h/d and the inactivated virus can both be recognized by anti-rH5HA1h anti-sera. The recognition sites of the anti-sera were mainly located in the peptide groups of HA-A, HA-B, and HA-E (Fig. 2).

Binding assays were also carried out using a neutralizing monoclonal antibody against H5N1 virus. Here, the samples were allowed to react with Y5 monoclonal antibody at different dilutions. In contrast to the results observed with the anti-rH5HA1h anti-sera, only the rH5HA1o and the inactivated virus were recognized by Y5 antibody in a dose-dependent manner (Fig. 3A). To further confirm that the binding of Y5 monoclonal antibody to rH5HA1o reflected the structural integrity of rH5HA1o, we performed competition assays. As shown in Fig. 3B, rH5HA1o but not rH5HA1d, rH5HA1h, nor the peptide pools can inhibit the binding of the Y5 monoclonal antibody to inactivated virus. With

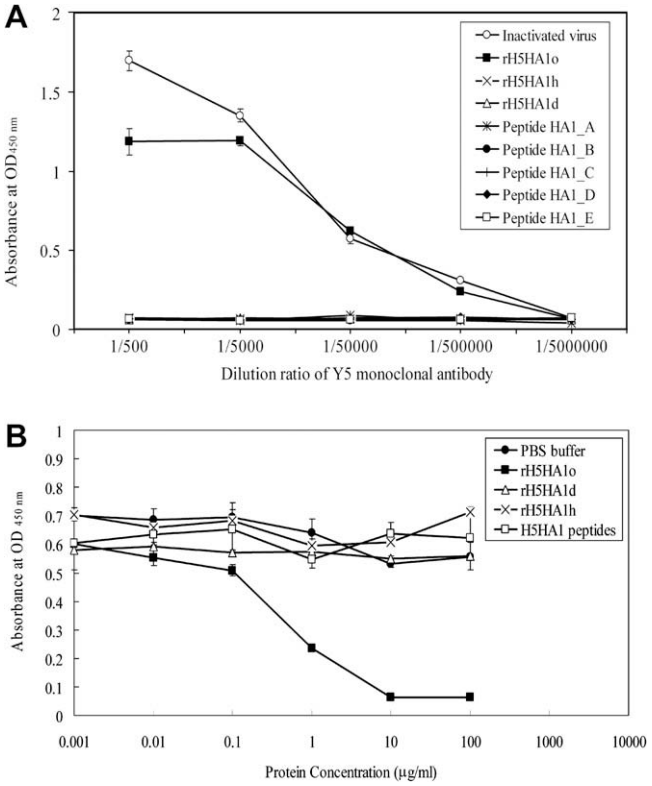

Fig. 3. Detection of the different forms of rH5HA1 by the neutralizing monoclonal antibody in a dose-dependant manner. (A) Different samples were coated at a concentration of 0.5 μg per well including peptide pools HA-A (-∗-), HA-B (-●-), HA-C (-+-), HA-D (-♦-) and HA-E (-□-). It also included rH5HA1 preparations rH5HA1d (-Δ-), rH5HA1h (-×-), rH5HA1o (-■-), and inactivated virus (-○-). The Y5 monoclonal antibody was diluted 1:500, 1:5000, 1:50,000, 1:500,000, and 1:5,000,000. The detection OD was 450 nm. As a control, the routine protocol was followed without coating protein onto the wells. These are represented in the graph as HRP_Y5 and HRP_Balb/c. The detection OD was 450 nm. (b) To perform the competition assay, the inactivated virus was coated in the wells and Y5 antibodies were diluted at 1/25,000 and incubated with different amounts of samples before reaction with inactivated virus. The samples included PBS (-●-), rH5HA1d (-Δ-), rH5HA1h (-×-), rH5HA1o (-■-) and H5HA1 peptides (-□-).

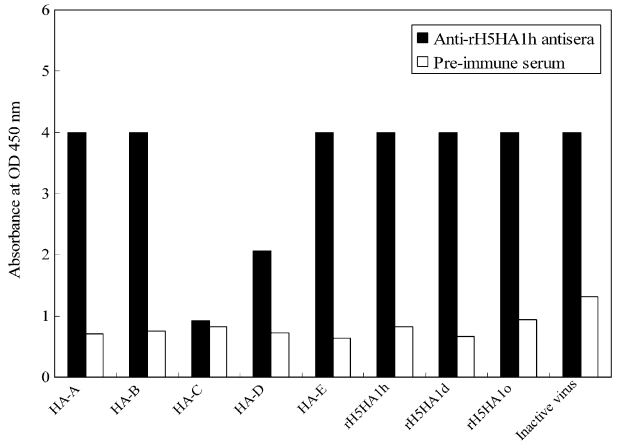

Fig. 2. Detection/recognition of different forms of H5HA1 by the rabbit polyclonal antibody to H5HA1. Different samples were coated at a concentration of 0.5 μg per well including HA-A to HA-E representing synthesized linear peptides covering the whole sequence of H5HA1 (see Table 1), rH5HA1 preparations obtained by using a high-pH buffer (rH5HA1h), detergent (rH5HA1d) or a redox buffer (rH5HA1o), or the inactivated virus. The antibodies used were the rabbit anti-rH5HA1h anti-sera (■) and pre-immune sera (□) and binding was detected using an HRP-conjugated anti-rabbit IgG as a secondary antibody with a colorimetric substrate measured at an absorbance of 450 nm.

the different concentrations of rH5HA1o, Y5 binding showed a typical competition profile, suggesting that the HA1 domain of HA can be refolded properly using the oxidative folding method. This result demonstrated that the rH5HA1o could compete with the binding of inactivated virus to Y5 antibodies and abolished the bound Y5 antibodies to the background value. Moreover, our on-column process dramatically increased the folding yield as compared with the conventional approaches in which samples are dialyzed against folding buffer in a very low concentration and for a much longer time.

The radii of isotopic spheres in rH5HA1 preparations are different

Previous data demonstrated that the folding state of rH5HA1 is different from other preparations. We further investigated the physical properties of rH5HA1 preparations using DLS. The results indicated that the hydrodynamic radius of rH5HA1d, rH5HA1h, and rH5HA1o are 3.4 nm, 9.7 nm, and 17.5 nm, respectively (Fig. 4). The distribution of rH5HA1d was a single peak and the polydispersity measured was 13.7% indicating that the rH5HA1d exists in solution predominantly in a single form. Although rH5HA1h and rH5HA1o contained two peaks, the dispersion of their major peaks (Ph I and Po I) were 12.7% and 17.7%, respectively, indicating that even though there were two peaks in

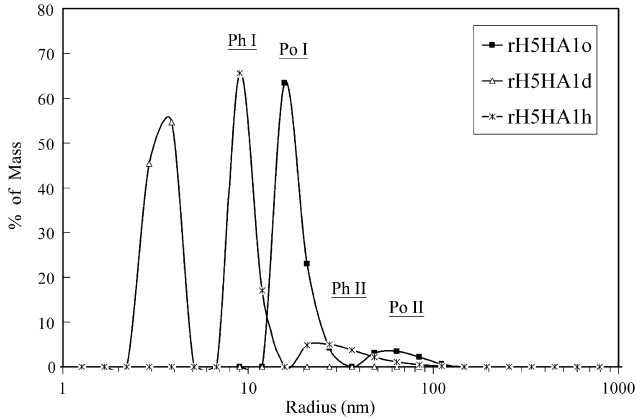

Fig. 4. Dynamic light scattering analysis of rH5HA1 preparations. rH5HA1s (rH5HA1o/h/d) were subjected to DLS at room temperature with a DynaPro 99 MS800 instrument. The individual DLS profile was exported to Microsoft Excel and combined all the profiles. The hydrodynamic radius of rH5HA1d (-Δ-) was a single peak. On the contrary, the rH5HA1h (-×-) contained two peaks (Ph I and Ph II) as did rH5HA1o (-■-) (Po I and Po II). The hydrodynamic radius of rH5HA1o is different from that of rH5HA1d and rH5HA1h.

rH5HA1h and rH5HA1o, these proteins still exist in a single form. These results suggested that the physical properties of rH5HA1o are different from that of other rH5HA1 preparations.

Discussion

The HA1 subunit of hemagglutinin contains most of its antigenic and neutralization sites. Despite its importance, there is currently a lack of information on the *in vitro* folding of this antigen. Considering that the HA1 domain contains nine cysteine residues and the recombinant protein frequently is found in inclusion bodies, the major challenge is obtaining an active protein during the refolding process [17]. Previously, folded HA/HA1 subunits or full length HAs derived from the Baculovirus expression system have been reported [18,19]. For the first time, we report here the production of an *E. coli*-derived soluble form of the HA1 of the influenza HA that can be recognized by a neutralizing monoclonal antibody. We thus demonstrate that the HA1 domain can be refolded even without post-translational modifications such as glycosylation.

We attempted to refold rH5HA1 using dialysis against buffer at neutral pH but this resulted in the massive aggregation of the protein. Although a detergent-based on-column folding protocol was also tried in this study, the resultant protein was not recognized by the Y5 monoclonal antibody. Our protocol did not use a urea gradient as most other groups have reported [14,20] but instead increased the volume of the folding buffer containing GSH/GSSG to increase the rate of protein oxidation [21] and thus prevent aggregation. The oxidatively-folded rH5HA1o was the only antigen to be recognized by the Y5 antibody. This result implies that there are conformational differences amongst the various forms of rH5HA1 that influence the recognition potential by the neutralizing monoclonal antibody. Thus, using an on-column folding protocol method, we obtained an H5-derived HA1 protein that was successfully folded as evidenced by its recognition by a neutralizing anti-H5 monoclonal antibody. This testifies to the fact that folding of most recombinant proteins should be approached on a case-by-case basis. We believe that our on-column strategy is a robust method to produce the antigen and thus envision strong potential for rH5HA1 as a vaccine candidate.

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