



Comparative Study of Methods for Extraction of Highly Purified Hemagglutinin from H5N1 Influenza Virus Recombinant Strain (A/Astana/6:2/2009)

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ABSTRACT

In the present study with the objective of the surface protein hemagglutinin production for single radial immunodiffusion reaction two methods of A/H5N1 recombinant strain A/AstanaRG/6:2/2009 highly purified hemagglutinin extraction were studied. They are ion exchange chromatography and adsorption on formalinized erythrocytes using such detergents as octyl glucoside, cetyl trimethylammonium bromide, X-100 triton. Homogeneous highly purified hemagglutinin has been obtained using the ion exchange chromatography.

KEYWORDS: Octyl glucoside, sucrose, concentration, protein, immunodiffusion, extraction, purification, ion exchange chromatography.

INTRODUCTION

In accordance with international and national standards hemagglutinin (HA) concentration is one of the most important requirements to quality of influenza vaccines [1, 2]. Expert committee on biological standardization (the WHO) recommended in its 29th report to show influenza vaccines efficiency in HA microgram per 1 ml (or dose) evaluated in single radial immunodiffusion reaction (SRIDR).

Pure HA and specific antihemagglutinin serum are important components for SRIDR. Standard antigen HA should meet the following requirements: to demonstrate homogeneity in electrophoresis in PAAG and to contain at least 100 µg of HA per 1 ml [3].

HA is a virus infection initiator at cell level, participating in virus particles adsorption by cell surface and genome penetration to cytoplasm, as well as the main target of immune response [4, 5, 6].

High structural variability of HA to a large extent determines periodic occurrence of new influenza epidemics and pandemics. These are the reasons why HA becomes one of the major objects for molecular, immunological, biochemical study, finally aimed at search for effective influenza control methods, as well as at development of optimal diagnosis, identification and control methods [7, 8].

Currently HA is isolated from influenza virions in several ways, one of which uses proteolytic enzyme bromelain. Till present day the best results were obtained by using bromelain (Sigma). However highly purified HA preparations obtained from many virus influenza strains with the help of bromelain (Sigma), have residual NA-activity. In case of using commercial bromelain preparations of other companies it is impossible to extract pure HA from influenza virions [9,10]. Influenza virus treatment often results in loss of neuraminidase (NA) and antigen activity of surface glycoprotein. Among cationic detergents only use of cetyl trimethylammonium bromide (CTAB) is rather successful.

There are data on the influenza virus glycoprotein extraction using detergents triton and cetylpyridinium chloride (CPC). The data show that when applying this method glycoprotein's yield is approximately 50-80%. However, during the process of viral glycoproteins obtaining detergent and phospholipid removal is a quite serious problem because during solubilization of virus membrane components phospholipids transform to solution to a great extent (~70% with triton and ~40% with CPC) [11].

Influenza virus processing leads to loss of neuraminidase and antigenic activity of surface glycoprotein. Among all cationic detergents cetyltrimethylammoniumbromide is successfully applied [12].

During the recent time huge attention has been focused on the method of chromatography. While affinity chromatography and detergent octyl-glucoside application HA output was from 30 to 84 % and NA activity was reduced to 0 [13].

For the destruction of influenza virions use a large number of detergents: nonidet - P40, Triton X-100, deoxycholate sodium, sarkozil NL30 and sodium dodecyl sulfate (SDS). Threshold concentration of micelle formation of first four detergents are sufficiently low and therefore used for virus destruction the aqueous solutions should not denature most

proteins. On the other hand, SDS is used in relatively high concentrations at which changes occur in the majority of denaturation of proteins. However, despite of this, the hemagglutinin of some virus strains retains its activity when contacted with SDS. One of the first methods for separation the hemagglutinin from neuraminidase without use a protease was based precisely on this high stability of hemagglutinin. Sodium dodecyl sulfate denatured the viral proteins, different from HA, which migrated at pH 9.0 as anions while hemagglutinin migrated as a cation. Thus, HA can be purified by electrophoresis on cellulose acetate. Unfortunately, this technique can not be used for all strains of influenza virus. Hemagglutinin of most strains denatures in the presence of SDS. When using other detergents neither hemagglutinin, not neuraminidase do not denature, but they can not be separated during electrophoresis. Hemagglutinin of these strains can be isolated in pure form only after the recombinants with SiDS-sensitive neuraminidase. In this regard, selection of hemagglutinin from most strains is quite challenging.

Thus, the main aim of our research is selection of optimal method for extraction of highly purified HA for SRIDR standard antigen preparation. And we obtained HA which corresponds to our SRIDR requirements.

Experimental procedures

Bioethics compliance

This study was carried out in compliance with national and international laws and guidelines on laboratory animal handling. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Research Institute for Biological Safety Problems Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (Permit Number: 0410/169).

MATERIALS AND METHODS

All works were carried out with observance of BSL-2, that is in a laminar flow of the second level of safety.

In experiences were used influenza virus recombinant strain A/AstanaRG/6:2/2009 (H5N1) received by reverse genetics method from the highly pathogenic influenza virus A/chicken/Astana/6/05 (H5N1) and donor-strain with high reproductive activity A/Puerto Rico/8/34 (H1N1). Developed domestic strain A/AstanaRG/6:2/2009 is reassortant in antigenic ratio of 6:2 between strains A/PR/8/34 (H1N1) and And/chicken/Astana/6/05 (H5N1), i.e. the recombinant strain consists from:

- six internal genes (PB2, PB1, PA, NP, M and NS), A/PR/8/34 (H1N1), received from a donor strain;
- two superficial proteins (HA and NA), received from a donor strain And/chicken/Astana/6/05 (H5N1).

Cultivation of (H5N1) influenza virus strain A/AstanaRG/6:2/2009 was carried out in chicken embryos. Virus containing allantoic liquid was inactivated by using of formalin.

Hemagglutinin extraction was made with two methods: ion-exchanged chromatography and adsorptions on formalinized erythrocytes.

HA extraction by methods of ion exchange chromatography

The preliminary clarification of the virus containing suspension was performed by centrifugation at $3,000 \times g$ for 20 min at a temperature of 4°C . Then the virus was treated with polyethylene glycol (PEG) – 8000 (Sigma - Aldrich chemie, Steinheim) to final concentration 8%. The concentrated virus was sedimented by centrifugation at $3000 \times g$ for 30 min a temperature of 4°C , the pellet was resuspended in 50 mM phosphate-buffered saline (PBS) ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) in volume 1/10 of the original one.

Purification and further virus concentration was performed by centrifugation at $124,500 \times g$ for 1 hour a temperature of 4°C through step gradient of 20-40-60% sucrose.

Virus fraction at 40% sucrose was centrifuged at $124,500 \times g$ for 1 hour a temperature of 4°C for sucrose removal. The sediment was resuspended with acetate buffer (50 mM CH_3COONa , 2 mM NaCl, 0,2 mM EDTA; pH=7,0; 7,25; 7,5) in volume 1/200 of the original one. HA and NA was extracted from virus membrane surfaces by treating the purified virus with non-ionic detergent n-octylglucoside (Calbochem-Behring corp. La-Jolla) to the final concentration 7% for 2 hours at 4°C at regular agitation [14]. After centrifugation at $27,340 \times g$ for 1 hour a temperature of 4°C the HA and NA containing supernatant was stored a temperature -20°C .

HA and NA were separated by the method of ion exchange chromatography. The HA- and NA containing supernatant was supplemented with 2% water solution of CTAB (Serva) to the final concentration 0.1% [12]. The sample was introduced into a column (1x3 cm) with sorbent DEAE-Sephadex A-50 (Pharmacia Fine Chemicals) preliminary balanced with start buffer (50 mM Tris-HCl, n-octylglucoside, pH= 7,0; 7,25; 7,5).

First NA was eluted in 20 ml of the start buffer and fractions were taken, and then HA was eluted in 20 ml of eluting buffer (50 mM Tris-HCl, 0,5 M NaCl, 0,1% Triton X-100, pH= 7,0; 7,25; 7,5) and fractions were collected. The eluting buffer contained Triton X-100 therefore every fraction was dialyzed against STE buffer for 72 hours a temperature of 4°C to remove residual n-octylglucoside and Triton X-100 (AppliChem, Darmstadt, Germany).

In HA fraction protein concentration was determined by Lowry method. Bovine serum albumin (BSA; Sigma-Aldrich, The Netherlands) was used as a standard. NA activity was determined by thiobarbituric method. Hemagglutinin activity was identified in hemagglutination reaction.

Virus proteins division and received preparations purity analysis have been carried out by means of electrophoresis in polyacrylamide gel with sodium dodecyl sulfate on the Laemmli method. Concentrating gels contained acrylamide in a concentration of 4 % and 10 % respectively. As a joining agent was used N - N- methylene-bis-acrylamide in a final concentrations 1/30 from acrylamide concentration. Electrophoresis was carried out during 5-6 hours at 25-30 mA. Albuminous strips have been revealed by means of Coomassie brilliant blue R-250 coloring (Pharmacia Biotech.). Electrophoregram analysis was carried out with the use computer program LabWork 4.0 for estimating of quantity and molecular masses of polypeptides. Proteins kit of the company Sigma was used as markers of molecular masses.

Electronic microscopy of the preparation was carried out by the method of negative contrasting with phosphotungstic acid. Increase x200000.

HA extraction by adsorption on formalized erythrocytes

The virus was concentrated in the following way: the virus-containing suspension was precipitated with PEG-6000 to a final concentration 7.5% at pH 8.5 for a day at a temperature of 4°C. The precipitated virus was collected 50 mM phosphate-buffered saline (PBS) (Na₂HPO₄*2H₂O, NaH₂PO₄*2H₂O) by centrifugation at 5.000 × g for 60 min at a temperature of 4°C. The virus was purified via ultracentrifugation at 10.000 × g for 60 min (4°C) followed by homogenization of the virus pellet till complete dilution and clarification by centrifugation at 5.000 × g for 10 min at a temperature of 4°C and the material was resuspended 50 mM phosphate-buffered saline (Na₂HPO₄*2H₂O, NaH₂PO₄*2H₂O).

The virus was destructed with Tween-80 in the final concentration 0.1% supplemented with ether in ratio 1:1 (volume/volume). The virions and nucleoproteins not destructed with Tween-ether, were removed by ultracentrifugation at 50.000 × g for 30 min at a temperature of 4°C and at 15.000 × g for 30 min respectively. The resulted supernatant (HA+NA) was dialyzed after ultracentrifugation at 150.000 × g at a temperature of 4°C against 10 mM solution of phosphate-buffered saline (0,145 M NaCl, 0,01 M KH₂PO₄) for 4 days at a temperature of 4°C with daily saline change.

The HA extracted by adsorption-elution with *formalized erythrocytes* rooster erythrocytes in the final concentration 10.0%. Protein content was also determined by Lowry method, neuraminidase activity by thiobarbituric acid method, HA activity in the hemagglutination reaction.

Purity and molecular weight of obtained HA were identified by electrophoresis in 10% PAAG.

Electronic microscopy of the preparation was carried out by the method of negative contrasting with phosphotungstic acid. Increase x200000.

RESULTS

Ion exchange chromatography

Experiments on HA extraction by ion exchange chromatography method were conducted. Destruction of virus by n-octylglucoside according to the method of Gallagher M. has shown that this detergent removes influenza virus glycoproteins from the virion surface. n-octylglucoside concentration in the course of the virus destruction was 7.0%. In further research the supernatant of surface glycoproteins solubilized with n-octylglucoside was divided by the method of ion exchange chromatography on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals) with use of cationic detergent CTAB (Serva).

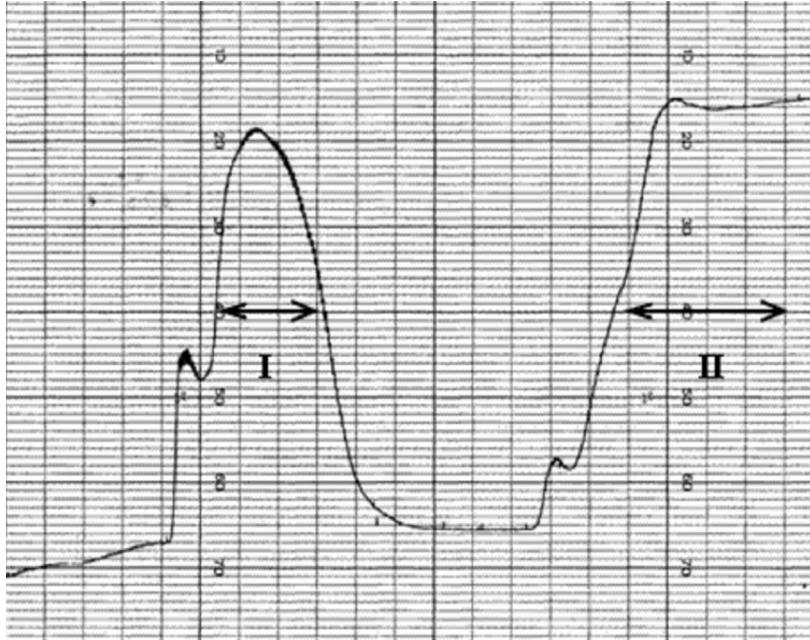
Since values of isoelectric points of NA and HA influenza virus may differ between the strains values of pH of the start and eluting buffers were selected experimentally. The buffers pH 7.0, pH 7.25, pH 7.5 were used to work with A/AstanaRG/6:2/2009 (H5N1) strain. Results of experiments are presented in Table 1.

Table 1 Results of HA extraction with the use of buffers with different pH values

Buffer, pH	Volume of obtained hemagglutinin, ml	Protein concentration, µg/ml	Hemagglutinin activity	Neuraminidase activity
7.0	10.0	400.0	1:512	0.00
7.25	10.0	300.0	1:256	0.00
7.5	10.0	300.0	1:128	0.00

It has been experimentally shown that use of start and eluting buffers, pH 7.0, is most optimal according to the parameters of protein concentration, hemagglutinin and neuraminidase activity.

Chromatographic analysis of the supernatant of influenza A/H5N1 virus A/AstanaRG/6:2/2009 surface HA and NA glycoprotein with use of buffer pH 7.0 is demonstrated in Fig. 1.

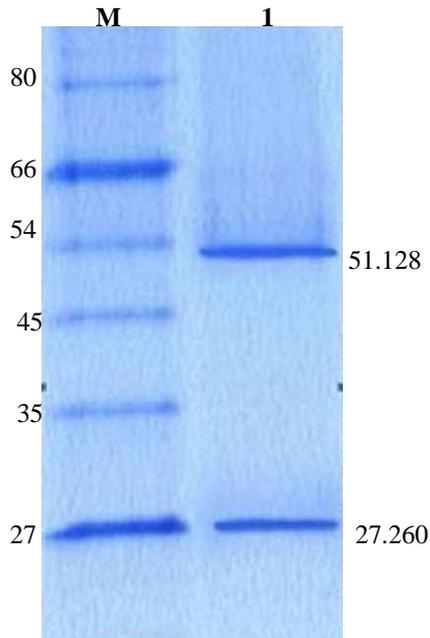


Peak 1 - neuraminidase fraction, peak 2 - hemagglutination fraction.

Figure 1 – Diagram of chromatographic division of HA and NA surficial glycoproteids supernatant of A/H5N1 influenza virus strain A/AstanaRG/6:2/2009

It is clear from figure 1 that the material leaving from a column was distributed in the form of two peaks of optical density where the first peak means an exit of a neuraminidase and the second peak an exit of hemagglutinin.

Purity and molecular weight of extracted HA of recombinant influenza virus A/AstanaRG/6:2/2009 (H5N1) strain was evaluated by means of electrophoresis in PAAG via comparison of its mobility with the same of marker (Sigma-Aldrich, The Netherlands) proteins. Figure 2 demonstrates the protein size which was counted using the software LabWorks 4.0.

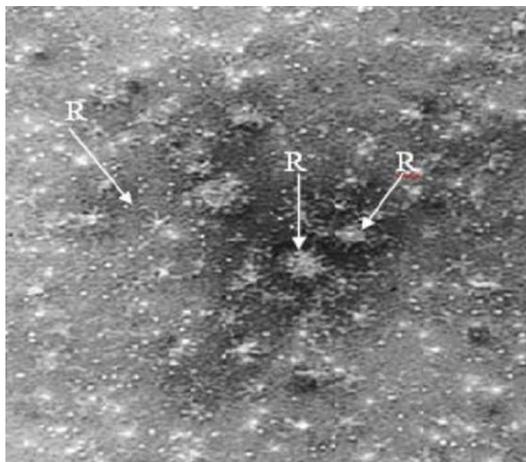


1 - HA fraction;

M – marker of molecular mass

Figure 2 – Electrophoretic analysis of extracted HA of (H5N1) influenza virus recombinant strain A/AstanaRG/6:2/2009.

Electrophoretic study of HA extracted by the method of ion exchange chromatography revealed two HA subunits with the molecular weights HA1=51.128 kDa and HA2=27.260 kDa. Depending on influenza virus strain its HA (molecular weight ~77.000 kDa) can be proteolytically split to two polypeptide chains: HA1 (~50.000 kDa) and HA2 (~27.000 kDa) which are connected with disulfide connections [15]. Electron microscopy of the resulted HA fraction (Fig. 3) has shown that in the preparation there are homogeneous structures as separate inclusions and structures in the form of rosettes (R).



Pointers presents HA, gathered in tufts.

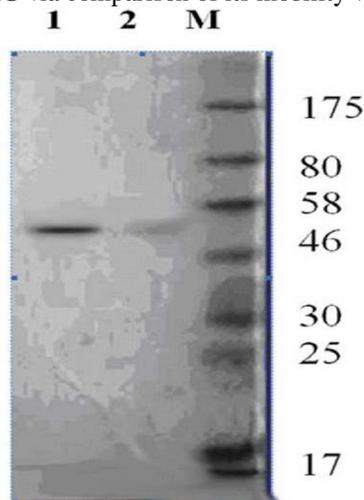
Figure 3 - Electronic microscopy of HA preparation of (H5N1) influenza virus strain A/AstanaRG/6:2/2009. Negative contrasting method with use of phosphotungstic acid. Increase x200000.

Data of electron microscopy of HA preparation of influenza A/H5N1 virus (A/AstanaRG/6:2/2009) was given in Fig.3. HA in the form of rosettes is shown by arrows. As it can be seen from the presented electron microscopy data, purification resulted in HA with connected hydrophobic ends forming rosettes.

Adsorption-elution with formalized erythrocytes

Further on HA was extracted by adsorption-elution with formalized erythrocytes. Tween-80 was used to destruct the virus [16]. The detergent detaches surface proteins that afterwards are sedimented by ultracentrifugation. The pellet contains undestructed surface proteins and nucleoproteins. HA was separated from other proteins by adsorption-elution with formalized erythrocytes. Hemagglutinin activity of extracted HA was 1:256, concentration of the total protein was 100 µg/ml, volume – 9.5 ml.

Purity and molecular weight of extracted HA of recombinant influenza (H5N1) virus A/AstanaRG/6:2/2009 strain were evaluated by electrophoresis in PAAG via comparison of its mobility with the same of marker proteins (Fig. 4).



1-2 – HA fractions;
M – marker of molecular mass.

Figure 4 – Electrophoretic analysis of extracted HA of (H5N1) influenza virus recombinant strain A/AstanaRG/6:2/2009.

The analysis showed that there were not any other virion components in the resulted HA preparation. However only HA1 associated with hemagglutinin activity is extracted by this method. The molecular weight of HA1 was 52.0 ± 0.375 kDa. Electron microscopy did not detect HA rosettes (Fig. 5).

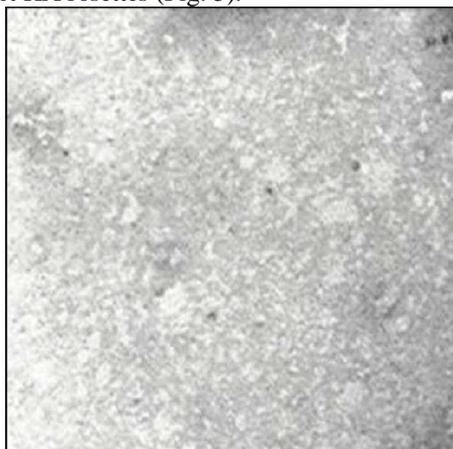


Figure 5– Electronic microscopy of HA preparation of (H5N1) influenza virus strain A/AstanaRG/6:2/2009. Negative contrasting method with use of phosphotungstic acid. Increase x200000.

Presented data on the electronic microscopy shows that in the result of purification there were no any remains and tufts of HA. And it means loss of HA during extraction process.

DISCUSSION

The major aim of the work was to compare two methods of extracting highly purified HA recombinant strain of influenza virus A/AstanaRG/6:2/2009 (H5N1) for its use in manufacture of the specific antihemagglutinin serum and antigen with known HA. Homogenous HA and specific antihemagglutinin serum are the necessary components for performance of the SRIDR. Separation of HA and NA surface proteins by the method of ion exchange chromatography with use of CTAB detergent results in their complete separation.

Homogeneity of the obtained HA was confirmed by the results of electrophoresis: only two subunits (HA1 and HA2) without any admixtures were clearly seen on the electrophoregram. The absence of neuraminidase activity in HA fractions confirms from findings of this study that there is no NA in the preparation. Electron microscopy of the obtained HA showed that the preparation contains HA in the form of rosettes.

In electrophoretic study of the HA extracted by adsorption-elution with formalized erythrocytes only HA1 subunit was demonstrated. This result was also confirmed by electron microscopy. The structure of this HA was destroyed; therefore it could not be used in production of the specific antihemagglutinin serum.

Activity of the HA extracted by the method of ion exchange chromatography was shown to be 1:512, that was twice higher as compared to activity of HA extracted by adsorption-elution method. The content of total protein was four times higher versus the HA extracted by the method of adsorption-elution with formalized erythrocytes.

Veselov [13] reported that during extraction of preparative influenza virus A/Brazil/11/78 (H1N1) hemagglutinin quantities when the virus was also destructed with n-octylglucoside detergent with subsequent HA extraction via affine chromatography the following results were obtained: volume of the material - 4 ml, protein concentration – 120 µg/ml, HA activity – 5000, NA activity - 0.00 [13].

The results of electrophoretic analysis of both strains confirms, that the HA is highly purified, separated from the NA component. Output HA strain A/Brazil/11/78 (H1N1) composed 54% and recombinant strain MRC-11 (H3N2) was 84%. According to the study [9] HA isolation performed by gelfiltration followed by ion exchange chromatography using two commercial grades bromelain "Sigma" and «Serva». Isolations highly purified HA two strains MRC-11 and A/SSSR/90/77 (H1N1) showed the following results NA activity was not detected, to yield highly purified HA strain MRC-11 was 67% and A/SSSR/90/77 strain (H1N1) 79%.

Comparison of these data with the results of using our method we can conclude that the method of ion exchange chromatography is preferential.

Analysis of the experiments allows to make conclusion that HA extracted by ion exchange chromatography is a highly purified homogenous preparation. Therefore this HA may be used in SRIDR.

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