Preparation of Neuraminidase specific antiserum from H9N2 subtype of avian influenza virus

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Abstract

Objective

In this research, affinity chromatography have been developed and standardized for production of Neuraminidase antigen of influenza virus for preparation of monospecific antiserum in rabbits.

Materials and Methods

Avian influenza Virus stocks (A/chicken/Iran/259/1998/(H_9N_2)) were propagated in the allantoic cavities of 10-day old embryonated chicken eggs. The harvested suspension was concentrated by polyethylenglycol 6000. Concentrated samples were layered onto sucrose gradient (30-60%). Both hemagglutinin and neuraminidase were solubilized from purified viruses with Triton X-100, across 30% sucrose gradient. NA was isolated from HA and other viral proteins by affinity chromatography on N- (p-aminophenyl) oxamic acid. Fractions that had high NA-activity and did not show HA activity were pooled and analyzed by neuraminidase inhibition and SDS-PAGE. For preparation of antisera, rabbits were immunized by purified NA and Freund's adjuvant at three weeks interval, and sera collected 7 days after boosting.

Results

In SDS-PAGE no viral protein band detected except for single band in the position of NA. NA activity of purified protein was 3.8×10^4 NA units. Enzymatic activity of Neuraminidase purified by this procedure decrease sharply above 48°C. The purified neuraminidase was producing a significant antibody response in agar gel precipitation. No reaction was observed with neuraminidase specific antiserum and H_9-HA of the same virus.

Conclusion

According to virtual purity and enzymatic activity of purified neuraminidase and highest avidity and specificity of antiserum, it was speculated that optimized protocol can be directly applied to produce antigen and antiserum from all subtypes of virus and can be easily used in commercial diagnostic tests.

Key words: Avian influenza, Neuraminidase, Antiserum.

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Introduction

Influenza A, B and C viruses are enveloped, segmented, single-stranded negative sense RNA viruses of the *Orthomyxoviridae*. Type A influenza viruses can infect several animal species, including birds, pigs, horses, seals and whales (1). Two types of glycoprotein projections cover the surface of Influenza A viruses: 1) rod-shaped trimers of hemagglutinin, and 2) mushroom-shaped tetramers of neuraminidase. At present fifteen hemagglutinin subtypes (H1–15) and nine neuraminidase subtypes (N1–9) of influenza A viruses have been recognized (2, 3). Hemagglutinin antigen (HA) and neuraminidase (NA), are the most important antigens since they are capable of stimulating protective immune response against infection (4).

The NA account is about 5-10 % of the virus protein and exists as spikes on the surface of the virion, possessing a hydrophilic area, which includes the active site, and a 'hydrophobic foot' that is inserted into the phospholipid bilayer surrounding the inner core of the influenza virion. This destroys neuraminic acid receptors on cell, and it aids release of the virus from the cells during replication, also antigenic, and plays a minor role in immunity to reinfection. This enzyme has been found in viruses, bacteria, protozoa and mammals shows remarkable differences according to the source (2).

The hemagglutinin and neuraminidase components may be separated from residual intact sub-viral particles using conventional methods for the separation of materials having different sizes or density, for example by gradient centrifuging, using sucrose or glutamate media, followed by fractionating of the gradients, sedimentation, molecular sieve chromatography or by pelleting in an ultracentrifuge (5).

In Iran, as in some other countries, avian influenza is an important infectious disease, every year affecting layer and breeder industries, usually fatally, and presenting a variety of unique challenges to animal health professionals and economic losses in poultry and turkey industry. A large number of laboratories in the worldwide are working in the surveillance of influenza virus activity and contribute to the early recognition of new emerging epidemic strains. Determination of the subtype of influenza A virus is the first step in the characterization of new isolated influenza viruses and is an essential part of early detection for prevention and eradication programs.

This is traditionally done by HI and Neuraminidase inhibition (NI) test with specific antisera raised in ferrets, sheep, rabbits or chickens (6). Unfortunately, monospecific antisera for avian influenza are commercially unavailable in the country, which are appropriate for use in diagnostic serological applications like NI and agar gel precipitation (AGP).

However, Affinity chromatography methods have been developed and standardized for the safe and reproducible production of neuraminidase antigen of influenza virus for preparation of monospecific antiserum in rabbits.

Purification of influenza viral NA must be preceded by solubilization either by the addition of detergents or proteolytic cleavage of the hydrophilic portion from the small hydrophobic foot (5). Many approaches have been used to purify the NA antigen (7). Other components may be isolated for further study by other methods (8-10).

Materials and Methods

Extraction and purification of HA and NA:
The virus A/chicken/Iran/259/1998/(H_9N_2) was grown in 10-day-old embryonated chicken eggs. The eggs were candled daily for embryo mortality, and allantoic fluid was clarified by absorption and elution from chicken erythrocytes followed by centrifugation at 3500 rpm for 15 min and

tested for hemagglutinin activity, PPLO and electron microscopy (EM). All viruses were frozen at −70 C until used.

The virus was concentrated by precipitation with 10% (w/v) polyethylene glycol 6000 and then purified by centrifugation at 9000 rpm for 50 min. Then, concentrated sample layered over an isopycnic (equilibrium) sucrose density gradient (30%-60%) that was made in a Beckman 25 X 76 mm ultraclear tube (Beckman Instruments, Inc., Fullerton, California) and centrifuged at 20000 rpm for 4h. Sucrose was removed using dialysis against Tris-Nacl-EDTA (TNE) buffer.

Triton X-100 was added to the virus concentrate in the form of an aqueous solution to a final concentration of 1-3% (v/v), which was then stirred and warmed at 30°C for 2h. The virus preparation was centrifuged at 20000 rpm for 2h to separating core proteins from surface proteins. The rich supernatant HA/NA was removed and the pH adjusted to 5.5 by using acetic acid.

Chromatographic separation of HA from NA: NA and HA were extracted from Triton X-100 and disrupted virus passed through an oxamic acid agarose column (11).

An N-(p-aminophenyl) oxamic acid (Sigma, St Louis, MO) agarose column was activated by washing with five column volumes of 0.1 M sodium bicarbonate solution containing 0.1% Triton X-100 and adjusted to pH 9.1, followed by five column volumes of 0.05 M sodium acetate solution containing 0.1% Triton X-100 and adjusted to pH 5.5. The HA/NA rich supernate was loaded on the column; the column was washed with 0.15 M sodium acetate solution, 0.1% Triton X-100, and pH 5.5, eluting the bulk of HA. Bound NA was eluted from the column with 0.1 M sodium bicarbonate solution, 0.1% Triton X-100, 0.002 M calcium chloride, pH 9.1(11). To rapidly lower the pH to which the purified enzyme is exposed, the effluent fractions are collected in tubes, which contain 0.5 M

sodium acetate buffer, pH 5.5, in a volume equal to 10% of the desired fraction volume.

After chromatography individual fractions were dialyzed against sodium acetate buffer (0.05 M sodium acetate, 2mM Nacl, 0.2mM EDTA (pH: 7.0)) for 72h to remove any residual detergent and each fraction was assayed for neuraminidase activity by employing fetuin and hemagglutinin activity (12). Fractions with optimal activity and the various preparations described above were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions by the method of O`Frarrell (13). Gels were silver stained by the method of Morrissey and analyzed to ensure that the preparations are free from proteins (14). Total protein in the preparation was measured by the method of Bradford (15).

Thermal and cryostability: NA sample was warmed at 1° per minute from 35-75°C. Aliquots were taken at 5°C intervals, rapidly cooled in an ice water bath and tested for NA enzymatic activity (16).

Preparation of anti neuraminidase sera: Rabbits (New Zealand White strain) were immunized by multi-site subcutaneous (s.c.) injection of 200 µg purified NA in Freund`s complete adjuvant.

At 21 days postinoculation they were boosted by subcutaneous injection with 100µg of NA in Freund`s incomplete adjuvant.

Seven days after the second injection, the rabbits were bled; sera collected for analysis by Agar gel precipitation (AGP) against purified NA, detergent-disrupted whole virus and purified H_9-HA of the same virus (12,15). When assays on test bleedings indicated that the serum had an adequate antibody titer and specificity, the animals were plasmasphered. Serum was recovered by incubation at 37°C until fully clotted, and extraction of the serum from the clot using a fruit press.

Results

Triton X-100 is a dialyzable, mild, nonionic detergent, which is capable of stripping surface antigens from the lipid bilayer (11, 17). The Triton X-100 supernatant was devoid of Matrix protein and contained most of HA and NA (Fig.1b). Protein recovery during the purification procedure is shown in Table, 55.1% of the total protein was solubilized by Triton X-100, and 8.3% of the total viral protein was associated with the NA peak obtained after oxamic acid affinity chromatography.
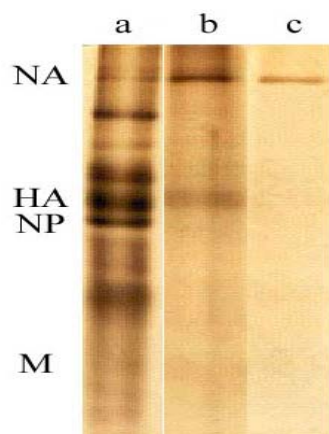

Figure 1. Analysis of influenza virus proteins with silver stain of 10% SDS-PAGE. Electrophoresis was performed under non-reducing condition.
a: A/chicken/Iran/259/1998/(H$_9$N$_2$) influenza virus, demonstrating the relative molecular weigh of influenza virus proteins, NP, 56 KDa; M$_1$, 27 KDa: NA, 220 KDa
b: Triton X-100 supernatant and
c: N$_2$-NA purified from Iranian avian influenza virus (H$_9$N$_2$).

Table. Protein recovery during NA purification from H$_9$N$_2$.

Purification stage	Protein Total (mg)	Recovery (%)
Total virus preparation	38.3	100
Supernatant	21.1	55.1
Oxamic acid Affinity chromatography	3.2	8.3

A slightly acidic buffer was used for the adsorption cycle, resulting in increased column capacity .The elution profile for the separation of N$_2$ NA from the H$_9$ HA on a N (p-aminophenyl) oxamic acid column is shown in Fig. 2.
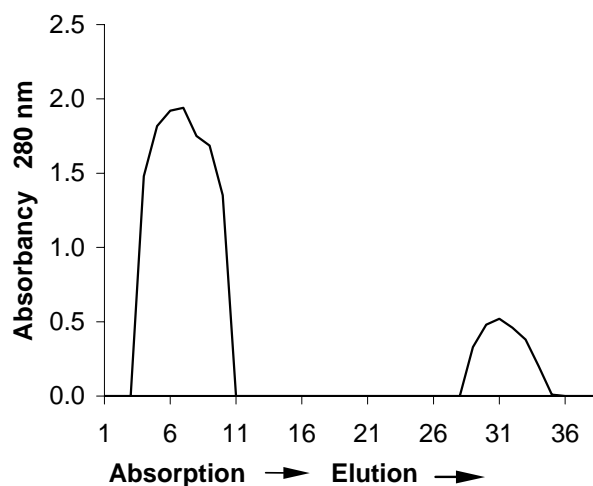

Figure 2. Affinity chromatography of Triton X-100 solubilized HA-NA from H$_9$N$_2$ virus preparation on N (p-aminophenyl) oxamic acid column.

Under the chromatographic conditions of the NA absorption buffer, the HA passed through the column in the exclusion volume and contained 93% of the sample protein and less than 8% of the sample NA activity. NA elution in the flow-through volume with no detectable hemagglutinin activity. Fractions from either source that had high NA activity (3.8×10^4 NA units) and did not show hemagglutination for chicken erythrocyte were pooled and analyzed by silver staining of SDS-PAGE. In this study SDS-PAGE was used for confirmation of the isolated NA.

On polyacrylamide gel electrophoresis as shown in Fig.1c, the neuraminidase migrated as a single high molecular weight component (220 KDa) and revealed no trace of other proteins. The heat stability of neuraminidases from influenza virus was shown to be variable (16). The results of the thermal stability and enzymatic activity of N$_2$ NA are shown in Fig. 3.
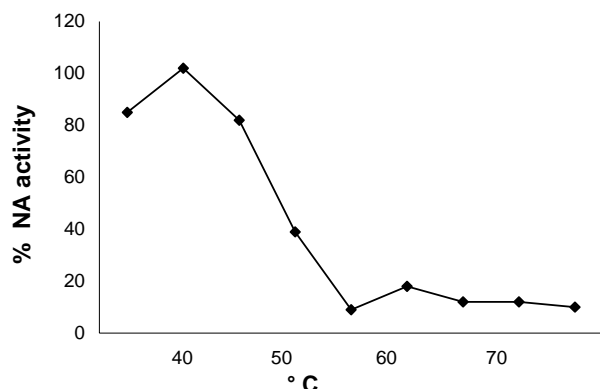
Figure 3. Thermal stability of purified neuraminidase. Sample was heated at 1°C per min and aliquots taken every 5°C from 35-75°C were tested for NA enzymatic activity.

In order to determine specificity and to assess the ultimate purity of NA, antisera were prepared against the neuraminidase in rabbits. Typical results shown in Fig. 4 demonstrate that isolated NA developed a single well-defined precipitin line against neuraminidase specific antiserum produced by the method represented in this paper. No reaction was observed with neuraminidase specific antiserum and H_9-HA of the same virus.
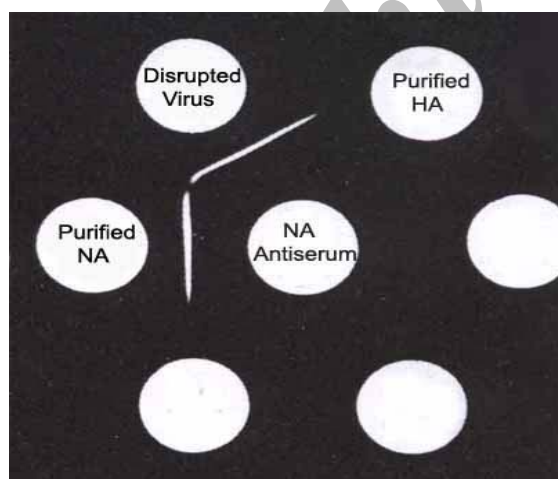

Figure 4. Agar gel precipitation of antisera prepared against isolated NA, HA of the same virus and disrupted H_9N_2 virus. Isolated NA and virus developed a single well-defined precipitin line against neuraminidase specific antiserum. No reaction was observed with neuraminidase specific antiserum and H_9-HA of the same virus.

Discussion

The neuraminidase purification technique described in this paper yields an enzymatically active neuraminidase (Fig. 3). Because both surface antigens belongs to membrane-associated glycoprotein with similar biochemical properties, separation of the two components is difficult, (7) and success of either procedure varies with different virus strains. No universally applicable method was found for the isolation of purified HA and NA antigens from all strains of influenza virus.

Separation of HA and NA molecules by chromatography is difficult because in their trimeric and tetrameric forms, HA and NA molecules, respectively, are close in molecular weight and have affinity for similar substrates. But it has been carried out both affinity (9, 10, 11, 12, 16) and ion exchange chromatographic procedures (7). Purification of NA or HA or both is dependent on development of conditions to exploit biochemical differences between two antigens. In the present study, satisfactory separation of the two major antigens was obtained by affinity chromatography .The use of N- (p-aminophenyl) oxamic acid agarose as a selective absorbent for influenza or bacterial neuraminidase has been demonstrated in other studies (5,11). Affinity chromatography separates NA from HA. More than 90% of the NA is removed from a typical HA-NA preparation. The collected NA in a single column cycle is virtually pure. This technique has been particularly successful for the purification of Neuraminidase, perhaps because these enzymes can be subjected to relatively rigorous conditions of detergent exposure without denaturation. The advantages of these procedures over the conventional methods of enzyme purification are including greater rapidity and yield, and a higher degree of purity.

The lack of hemaggluting activity in the purified virus enzymes, further indicate that neuraminidase and hemagglutinin are separated. The use of Triton X-100 stabilizes and maintains the solubility of neuraminidase and retains its activity. We found, that detergent concentration as high as 3%, NA and HA were fully dissociated from one another. Also; $Ca2^+$ in dialysis buffer stabilizes viral NA enzymatic activity (18), and immunogenicity (E.D.Kilbourne, unpublished results). However, the enzyme appeared to be equally stable with or without additional Triton X-100 (5).

Enzymatic activity of Neuraminidase purified by this procedure decrease sharply above 48°C (Fig. 3). Only a few attempts have been made to determine the subtypes of influenza A viruses by methods other than HI and NI either after passage in cell culture or directly from clinical specimens. More recently, the reverse transcriptase PCR method has been applied for the type-specific and subtype-specific detection of influenza virus RNA in clinical specimens (19).

Antisera to eight of nine NA subtypes of reference influenza A antigens have been prepared in goats (12). These reference antisera were prepared using purified preparations of NA and are considered monospecific. In the cases where monospecific antisera not available, polyclonal antisera to the reference virus has been prepared. Reference antisera are designed to distinguish between different NA subtypes but are broadly cross-reactive to detect as many different variants as possible within a certain NA subtype (12).

The antigens are analyzed for their ability to induce specific antibody formation in rabbits. Antisera raised against purified NA by the method described in this report give reactions to NA and disrupted H_9N_2 virus in AGP (Fig 4). The results indicate that the monospecific anti-HA antiserum neither neutralized the NA activity nor gave a precipitation line by AGP test against NA. The antiserum produced by injection of purified viral HA (unpublished results) neutralized HA activity and gave precipitin lines when AGP tests were performed, and did not cross-reaction against viral NA.We have also observed that highly specific NA antiserum was a valuable experimental tool in determining of significance function or the role of enzyme during virus multiplication (unpublished results).

We have demonstrated that the NA antiserum produced by this method, are high quality reagents and suitable for serological applications in the diagnostic laboratory.

The present rapid, simple and efficient method for preparation of NA from influenza virus should be useful not only in producing live NA-specific vaccines based upon purified NA, against highly pathogenic avian influenza but also for the preparation of NA for study of structure activity. Also, The antigens are suitable for use in either Neuraminidase inhibition and agar gel precipitation and the optimized protocols can be directly applied to produce antigens from new or emerging influenza viruses.

The principal advantage of N-specific vaccines is that they do not interfere with serological diagnosis and monitoring by HI tests, thus it would be possible by HI testing to distinguish between vaccinated flocks which had not received a field challenge and those which had. A study of efficiency of N_2 –specific vaccines in chickens and detection of Neuraminidase specific antibody by ELISA and immunobloting procedures are currently being undertaken.

Acknowledgments

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