Preparation of Neuraminidase-Specific Antiserum from the H9N2 Subtype of Avian Influenza Virus

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Abstract: Avian influenza virus stocks (A/chicken/Iran/259/1998/(H9N2)) were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs. The harvested suspension was concentrated by polyethylene glycol 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. No viral protein bands were detected, except for a 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 decreased sharply above 48 °C. For preparation of antisera, rabbits were immunized with purified NA and Freund's adjuvant at 3-week intervals, and sera were collected 7 days after boosting. The purified neuraminidase produced a significant antibody response in agar gel precipitation. No reaction was observed with neuraminidase-specific antiserum or H9-HA of the same virus.

Key Words: Avian influenza, neuraminidase, antiserum

Introduction

There are 2 types of glycoprotein projections that cover the surface of influenza A viruses: 1) rod-shaped trimers of hemagglutinin, and 2) mushroom-shaped tetramers of neuraminidase. At present, 15 hemagglutinin (HA) subtypes (H1-15) and 9 neuraminidase (NA) subtypes (N1-9) of influenza A viruses have been identified (1-3). NA accounts for about 5%-10% of the virus protein, exists as spikes on the surface of the virion, and possesses a hydrophilic area (2,4).

HA and NA 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).

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 HA inhibition (HI) and NA inhibition (NI) tests using specific antisera raised in ferrets, sheep, rabbits, or chickens (6).

Unfortunately, monospecific antisera for avian influenza that are appropriate for use in diagnostic serological applications like NI and agar gel precipitation (AGP) are commercially unavailable in Iran. However, affinity chromatography methods have been developed and standardized for the safe and reproducible production of the NA antigen of influenza virus for the preparation of monospecific antiserum in rabbits.

Many approaches have been used to purifying the NA antigen (7). Other components may be isolated for further study of other methods (8-10).

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Materials and Methods

Extraction and purification of HA and NA

The virus, A/chicken/Iran/259/1998/(H9N2), was grown in 10-day-old embryonated chicken eggs. The eggs were candled daily to check for embryo mortality, and allantoic fluid was clarified by absorption and elution from chicken erythrocytes, followed by centrifugation at 3500 rpm for 15 min. Then the fluids were tested for the presence of influenza virus and differentiated from other HA positive microorganisms by the hemagglutination inhibition test and electron microscopy (EM), and differentiated from *Mycoplasma* spp. by the PPLO test. All viruses were frozen at –70 °C until use.

The virus was concentrated by precipitation with 10% (w/v) polyethylene glycol 6000 and then purified by centrifugation at 9000 rpm for 50 min. Subsequently, a concentrated sample layered over an isopycnic (equilibrium) sucrose density gradient (30%-60%) was made in a Beckman 25 × 76 mm ultraclear tube (Beckman Instruments, Inc., Fullerton, CA, USA) and centrifuged at 20,000 rpm for 4 h. Sucrose was removed using dialysis against Tris-NaCl-EDTA (TNE) buffer. Then Triton X-100 was added to the virus concentrate in the form of an aqueous solution to make a final concentration of 1%-3% (v/v), which was then stirred and warmed at 30 °C for 2 h.

The virus preparation was centrifuged at 20,000 rpm for 2 h to separate core proteins from surface proteins. The rich supernatant HA/NA was removed and the pH adjusted to 5.5 using acetic acid.

Chromatographic separation of HA from NA

NA and HA were extracted from Triton X-100 and the disrupted virus was 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 5 column volumes of 0.1 M sodium bicarbonate solution containing 0.1% Triton X-100 adjusted to pH 9.1, followed by 5 column volumes of 0.05 M sodium acetate solution containing 0.1% Triton X-100 adjusted to pH 5.5. The HA/NA-rich supernate was loaded on the column, and the column was washed with 0.15 M sodium acetate solution and 0.1% Triton X-100 (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, and 0.002 M CaCl (pH 9.1) (11).

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

Thermal and cryostability

The NA sample was warmed at the rate of $1° \, min^{-1}$ 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 injection of 200 µg purified NA in Freund's complete adjuvant. At 21 days post-inoculation they were boosted by subcutaneous injection with 100 µg NA in Freund's incomplete adjuvant. The rabbits were bled 7 days after the second injection; sera were collected for analysis by agar gel precipitation (AGP) against purified NA, detergent-disrupted whole virus, and purified H9-HA of the same virus (12,15).

Results

The Triton X-100 supernatant was devoid of matrix protein and contained most of the HA and NA (Figure 1b). Protein recovery during the purification procedure is shown in the 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.

A slightly acidic buffer was used for the adsorption cycle, resulting in increased column capacity. The elution profile for the separation of N2 NA from H9 HA on an N-(p-aminophenyl)oxamic acid column is shown in Figure 2.
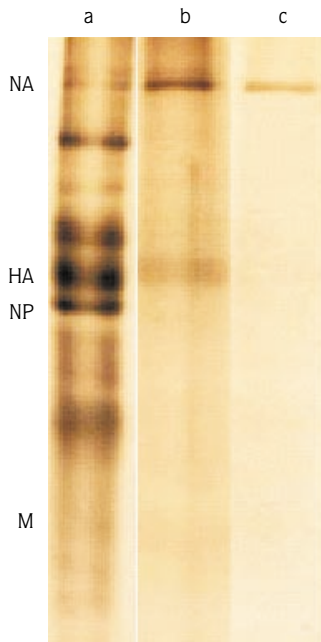
Figure 1. Analysis of influenza virus proteins with silver staining of 10% SDS-PAGE. Electrophoresis was performed under non-reducing conditions.

a. A/chicken/Iran/259/1998/(H9N2) influenza virus, demonstrating the relative molecular weight of influenza virus proteins, NP, 56 KDa; M_1, 27 KDa: NA, 220 KDa.
b. Triton X-100 supernatant.
c. N2-NA purified from Iranian avian influenza virus (H9N2).

Table. Protein recovery during NA purification from H9N2.

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

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 < 8% of the sample NA activity. NA was eluted 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.
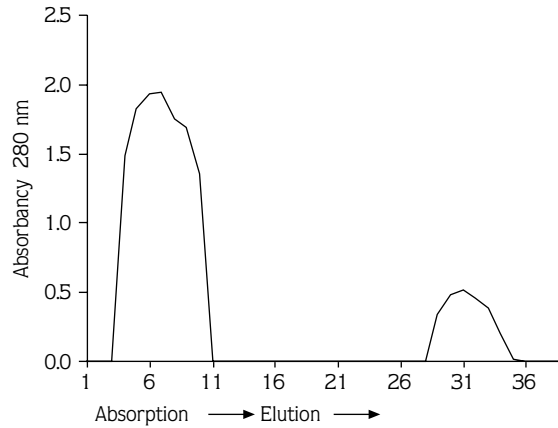

Figure 2. Affinity chromatography of Triton X-100 solubilized HA-NA from H9N2 virus preparation on an N-(p-aminophenyl) oxamic acid column.

In this study SDS-PAGE was used for confirmation of the isolated NA. With polyacrylamide gel electrophoresis, as shown in Figure 1c, NA migrated as a single high molecular weight component (220 kDa) and revealed no trace of other proteins.

The thermal stability and enzymatic activity of N2NA are shown in Figure 3.

In order to determine specificity and to assess the ultimate purity of NA, antisera were prepared against NA in rabbits. Typical results shown in Figure 4 demonstrate that isolated NA developed a single well-defined precipitin line against NA-specific antiserum produced by the method represented in this paper. No reaction was observed with NA-specific antiserum and H9-HA of the same virus.

Discussion

The neuraminidase purification technique described in this paper yields an enzymatically active NA (Figure 3). Because both surface antigens belong to membrane-associated glycoprotein with similar biochemical properties, separation of the 2 components is difficult (7,17) 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.
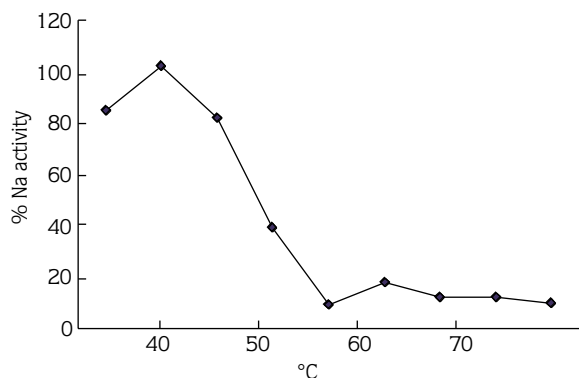
Figure 3. Thermal stability of purified NA. The sample was heated at the rate of 1 °C min-1 and aliquots were taken every 5 °C from 35-75 °C were tested for NA enzymatic activity.
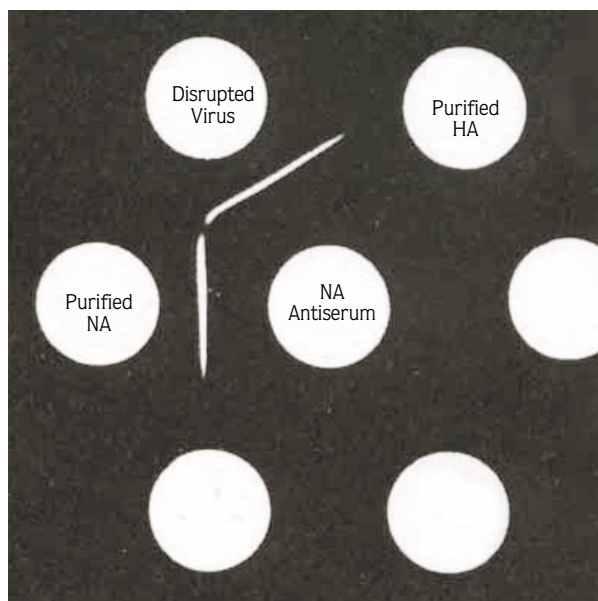

Figure 4. Agar gel precipitation of antisera prepared against isolated NA, HA of the same virus, and disrupted H9N2 virus. Isolated NA and the virus developed a single, well-defined precipitin line against NA-specific antiserum. No reaction was observed with NA-specific antiserum and H9-HA of the same virus.

Separation of HA and NA molecules with 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. Nevertheless, it has been carried out, both with affinity (9-12,16) and ion exchange chromatographic procedures (7). Purification of NA, HA, or both is dependent on the development of conditions to exploit biochemical differences between 2 antigens. In the present study satisfactory separation of the 2 major antigens was obtained by affinity chromatography. The use of N-(p-aminophenyl)oxamic acid agarose as a selective absorbent for influenza or bacterial NA has been demonstrated in other studies (5,11). Affinity chromatography separates NA from HA. More than 90% of 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 NA, 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 include greater speed and yield, and a higher degree of purity.

The lack of HA activity in the purified virus enzymes further indicates that NA and HA are separated. The use of Triton X-100 stabilizes and maintains the solubility of NA and retains its activity. We found that with detergent concentration as high as 3%, NA and HA were fully dissociated from one another. Moreover, Ca^{2+} 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 NA purified by this procedure decreased sharply above 48 °C (Figure 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).

The antigens were analyzed for their ability to induce specific antibody formation in rabbits. Antisera raised against purified NA by the method described in this report gave reactions to NA and disrupted H9N2 virus in AGP (Figure 4). The results indicate that the monospecific anti-HA antiserum neither neutralized NA activity nor gave a precipitation line by the 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 there was no cross-reaction against viral NA.

We also observed that highly specific NA antiserum was a valuable experimental tool in determining the significance of function or the role of the enzyme during virus multiplication (unpublished results).

We demonstrated that the NA antisera 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 the study of structure activity. Furthermore, the antigens are suitable for use in either NI or agar gel precipitation and the optimized protocols can be directly applied to produce antigens from new or emerging influenza viruses. A study of the efficiency of N2-specific vaccines in chickens and the detection of NA-specific antibody by ELISA and immunoblotting procedures are currently being undertaken.

Acknowledgments

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