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ISOLATION OF HEMAGGLUTININ WITH NEURAMINIDASE ACTIVITY FROM INFLUENZA A/AICHI/2/68 (H3N2) STRAIN

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ABSTRACT

When the influenza virus samples, which were purified and treated with ether containing 0.05% Tween-80, were applied repeatedly to fetuin-ligand affinity chromatography, HA was highly purified. The sample from the Aichi strain (H3N2) gave 2 peaks after elution of the affininty column. The fractions from the first one showed a 1: 1 ratio of HA to NA activities, an abnormally high NA activity. These fractions also showed lower reactivity with the anti HA and NA antisera. These suggested that, although there is no difference between the antigenicities of the purified sample and the original sample, the HA and the NA exert steric inhibition on each other.

INTRODUCTION

Influenza type A virus particles have two different glycoproteins, hemagglutinin (HA) and neuraminidase (NA), on their surface. The antigenic shift occurring periodically causes the subtyping of HA into H1, H2 and H3, and NA into N1 and N2 (Stuart-Harris and Schild, 1976).

The influenza HA monomer is first synthesized as a single polypeptide chain which

undergoes post-translational proteolysis to remove its N-terminal signal sequence and cleave the molecule into two polypeptide chains called HA1 and HA2. HA1 and HA2 remain joined by a disulfide bond and each HA spike is a trimer of these HA1 and HA2 chains (Laver, 1973). HA is resposible for the attachment of virus particle to cell membrane receptor molecules which contain sialic acid. This interaction has been shown to vary in detailed specificity for different influenza viruses (Carroll et al., 1981). HA also possesses pH-dependent fusion ability which plays a key role in uncoating during the initial stages of infection (Maeda and Ohnishi, 1980; Huang et al., 1981; White et al., 1981).

On the other hand, the role of NA in the infection process is not clear. This enzyme exists as a mushroom-shaped spike on the surface of the virus particle and catalyses cleavage in the linkage between terminal sialic acid and adjacent sugar residue. This reaction allows the release of progeny virus particles from infected cells (Colman and Ward, 1985). At the same time, NA is known to remove sialic acid from the carbohydrate moiety of newly synthesized hemagglutinin, a reaction which is necessary to prevent self-aggregation of the virus (Palease et al., 1974).

At present, many works can be found on the function of antigenic shift occurring periodically at the molecular level using the technique of sequencing the genes which code HA (Porter et al., 1979; Gething et al., 1980; Jou et al., 1980; Lai et al., 1980) and monoclonal antibodies to the antigenic determinants (Caton et al., 1982; Nakajima et al., 1983). We are now trying to produce monoclonal antibodies against the HA of each subtype of influenza virus. The monoclonal antibodies should have the important characteristics of more effectively estimating the antigenic determinants on the HA molecules, the HA activity and the region which shows pH-dependent fusion activity. For this purpose, it is essential to prepare a purified HA sample. We have applied fetuin-ligand affinity chromatography for purification of the HA, and used the purified HA as an antigen in hemagglutination inhibition (HI) test by the homologous antibody. The result was that purified HA fraction lowered an HI titer in the subtype of H3N2 compared with whole virion as an antigen, but in the subtypes of H1N1 and H2N2 , suggesting that the action could be derived from the steric inhibition of NA contaminated in the purified HA fraction.

In this paper, we describe the difference of behavior on the fetuin-lignad affinity chromatogram and immunoreactivity to the homologous antibodies among the H1N1, H2N2, and H3N2 subtypes.

MATERIALS AND METHODS

Viruses. Influenza viruses used in this study were A/Adachi/2/57 (H2N2), A/Aichi/2/68(H3N2),A/Tokyo/6/73(H3N2),A/Niigata/1/75(H3N2)andA/USSR/92/77(H1N1). Seed stocks of viruses were inoculated in the allantoic sac of 10-day embryonated chicken eggs and were grown for 3 days at 35℃. Viruses were purified by sucrose-

density gradient according to Laver (1969).

Affinity chromatography for purification for HA. Affinity chromatography was performed by the method of Becht and Rott (1972) to purify the HA. This method was based on the affinity of HA and NA molecules to fetuin, which was named by Pederson and is a general name for serum protein of m. w. of ca, 48,000 daltons and contains ca. 8% N-acetylneuraminic acid (Graham, 1972). Also, that the nature of NA is protein which shows no enzymatic activity at state of cooling is assumed in this method. Samples were prepared by shaking the purified virus, suspended in equal volume of PBS and ehter containing 0.05% Tween-80, for 30 min at room temperature, centrifuging for 15 min at 2,800rpm and treating the resulting aqueous layer with ether. Fetuin was obtained from Gibco. CNBr-activated Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden.

Affinity chromatography for purification of NA. NA was purified using N- (p -aminophenyl) oxamic acid-ligand affinity chromatography according to Cuatrecasas (1973). Sepharose coupled with a specific inhibitor of NA, N- (p-aminophenyl) oxamic acid, was obtained commercially from Sigma, St. Louis, MO, U. S. A.

HA and HI tests. The measurement of HA and HI titers was carried out by the standard micro method (Sever, 1962).

NA and NI tests. The activity of NA and NI titer were measured by the method recommended by W. H. O. (Aymard-Henry et al., 1973).

Preparation of antiserum. The antiserum against whole virion was prepared by the injection of infected allantoic fluid (1024 HA) into chicken (5 ml i. p. and 0.5 ml i. v.) and a booster shot (i. v.) of 0.5 ml, 1 ml and 2 ml of the fluid every 4 days. The antiserum against the fraction obtained from affinity chromatogrphy was prepared by immunizing the chicken with the injection of 1-2 ml of each fraction diluted with PBS of up to 0.1-1 mg of protein per ml into the wing vein and abdominal cavity once a week for 4 weeks. Four days after the last treatment, the blood was collected and the serum was separated by centrifugation and stored at-20℃.

RESULTS

The purification of HA from the ether-treated virus samples was carried out by fetuin-ligand affinity chromatography (Fig. 1). The recovery rate of HA was 90-95% in all influenza virus strains. In both strains of USSR and Adachi, the HA fraction was contaminated with NA (about 10 % of total NA activity) , but the NA could be removed by passing it repeatedly through column or by performing affinity chromatography again for its purification. In the Aichi strain, two peaks with close elution time were observed, the first one showing a higher NA activity and lower HA activity compared with the latter one. All eluted fractions in the Aichi strain were contaminated with 32 % of all the NA activity, and this ratio was higher than that in the USSR or Adachi strains.

The fractions which showed HA activity were used as antigens for the HI test with

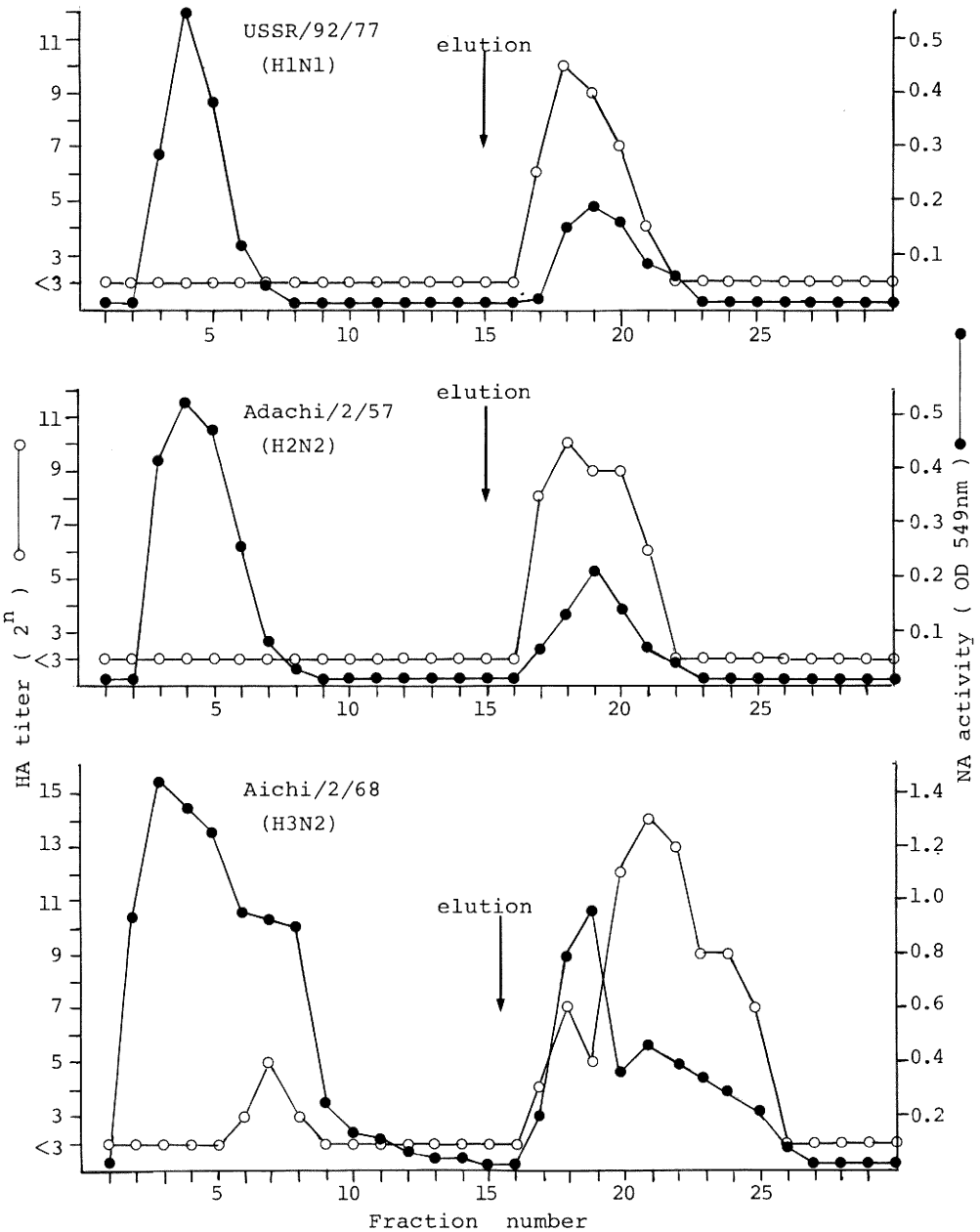

Fig. 1. Fetuin-ligand affinity chromatography. Ether-treated virus samples were applied to column, unadsorbed materials were rinsed off with BKE buffer (0.1 M boric acid-tris, pH 8.2 containing 0.1 M KCl and 2 mM EDTA) at 4℃ and then adsorbed materials were eluted with BKE + 3 M KCl at 37℃ (arrow). The size of each fraction is 2 ml.

Table 1. HI test.

	H1N1	H2N2	H3N2		
	USSR/92/77	Adachi/2/57	Adachi/2/68	Tokyo/6/73	Niigata/1/75
Whole virion	2048	2048	2048	2048	2048
Ether treatment	4096	4096	4096	4096	4096
Fraction number of fetuin affinity chromatography					
Pre-elution. No.6			4096		
7			4096		
8			4096		
Elution. No.17	4096	4096	512		
18	4096	4096	512	256	256
19	4096	4096	256		
20	4096	4096	4096		
21		4096	4096		
22		4096	4096	4096	4096
23			4096		
24			4096		
25			4096		

Table 2. Ratio of HA and NA activity (Aichi/2/68).

	HA	NA
Whole virion	12.5	1.0
Fraction no. 7	0.48	1.0
no.18	1.0	1.0
no.22	142.0	1.0

homologous antiserum. Tabie 1 shows the results of the HI test using the whole virion and ether-treated samples as antigens. Both USSR and Adachi strains showed the same HI patters, and the HI titers of ether-treated samples and all the fractions tested were twice as high as those of the whole virion in these strains. On the other hand, in the Aichi strain, fraction No. 17-19, corresponding to an earlier peak of eluted fractions, showed quite different HI titers that were 4-8 times lower than those of the whole virion. Therefore, two strains of H3N2 subtype (Tokyo/6/73 and Niigata/1/75) were subjected to further experiment in the same way as mentioned above, showing the same results as seen in the Aichi strain (Table 1.). These results indicated that this phenomenon should be specific for each H3N2 subtype. In order to make this clear, the following experiment was performed using the Aichi strain.

The ratio of HA in each sample is shown in Table 2. The titer of NA is expressed as reciprocal of the dilution titer which showed an absorption of 0.1 at 549nm. In Table 2, the activity of HA calculated by this method is designated as 1 to obtain the relative activity of HA. The HA titers of whole virion, fraction No. 7 and No. 22 were 12.5, 0.48

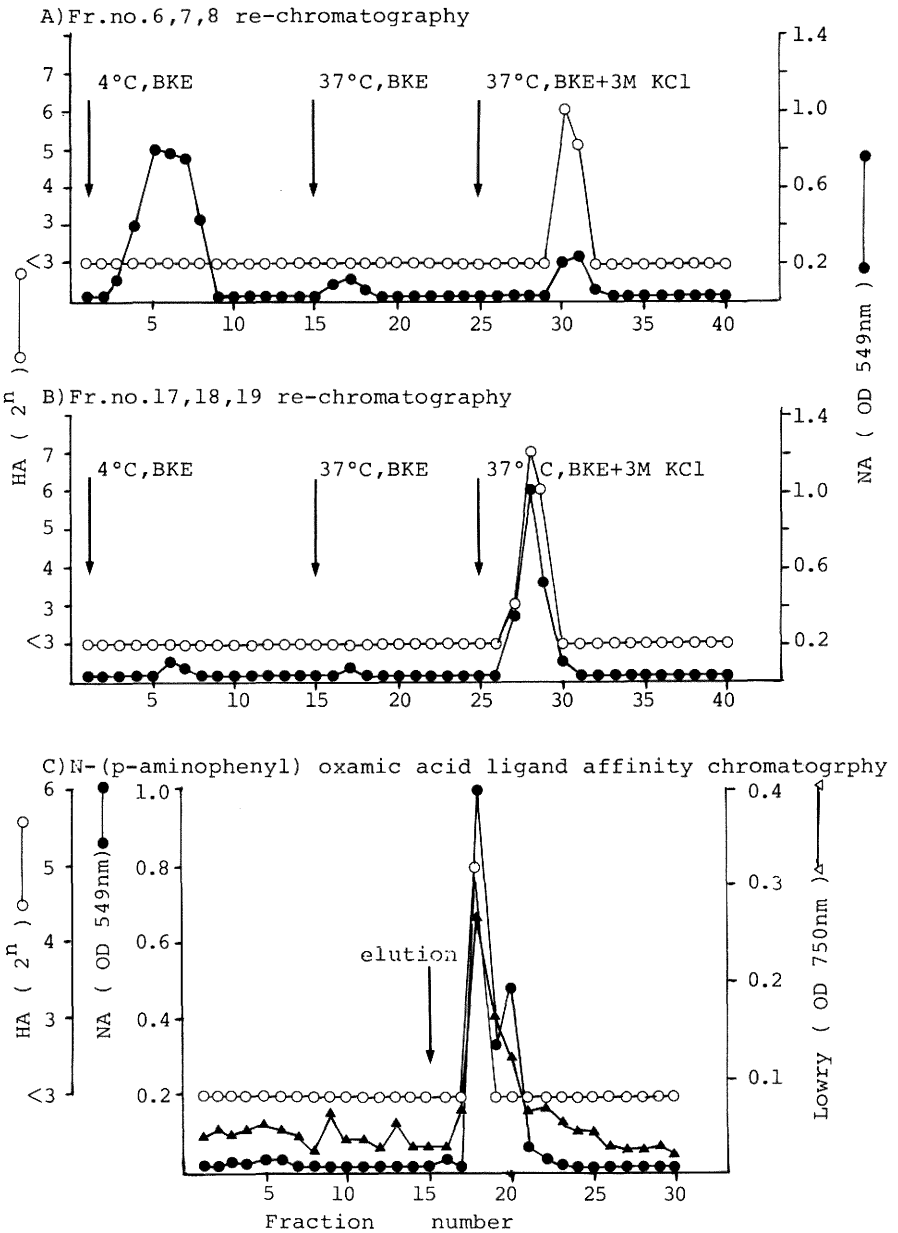

Fig. 2. Re-chromatography of the pass-through fraction and the BKE + 3 M KCl eluate of Aichi strain (see Fig. 1) and N-(p-aminophenyl) oxamic acid-ligand affinity chromatograpgy. The separation of HA and NA in the Aichi strain was attempted by re-chromatography of the jointed solution of fraction no. 6-8 (A) and no. 17-19 (B). Buffers for elution were indicated in figure. Furthermore, the fraction no. 26-30 in Fig. 2 (B) were subjected to N- (p-aminophenyl) oxamic acid affinity column for the separation of NA using 50 mM sodium acetate, pH5.5 containing 2 mM $CaCl_2$, 0.2 mM EDTA and 0.1% Triton X -100 as a washing buffer and 0.1 M bicarbonate buffer, pH 9.1 as an elution buffer (C). The size of each fraction is 2 ml. The eluate was collected in the tube containing 0.2 ml of 0.5 M sodium acetate buffer, pH 5.0.

Table 3. HI and NI tests.

Antibody	Antigen				
	Whole virion	Pre-elution	Pre-elution re-chromato.	HA-NA	HA-NA re-chromato.
Anti whole virion	2048 (460.8)	2048 (460.8)	2048	512 (147.6)	256
Anti HA-NA	4096 (46.4)	4096 (46.4)	4096	8192 (64.0)	16384

( ) : NI test.

and 142.0, respectively. The relative activity of HA to NA in fraction No. 22 was about 11 times higher than that of the whole virion. On the other hand, fraction No. 18 was in a ratio of 1: 1, showing an abnormally high activity of NA.

The separation of HA and NA in the Aichi strain was attempted by rechromatography of the jointed solution of fraction No. 17-19 in the same manner as in the USSR and Adachi strains. First, the temperature of the washing buffer was shifted from 4℃ to 37℃, before changing the washing buffer to elution buffer, to facilitate NA elution. Slight peaks of NA could be seen by using the washing bufter at 4℃ and 37℃; however, the large majority of NA activity was detected and was impossible to separate from HA protein (Fig. 2B). Therefore, the fractions of No.26-30 (in Fig. 2B) obtained by re-chromatography were subjected to affinity chromatography for the purification of NA, in expectation of the adsorption of NA on the ligand and the recovery of HA from its passing through the solution (Fig 2C). But, its was impossible to remove NA from HA fraction because NA was also recovered in the same fraction as the HA fraction.

Subsequently, the antisera against HA-NA (Fr. No. 26-30 in Fig. 2B), re-chromatographed HA-NA (Fr. No. 17-19 in Fig. 2C), pre-eluted (Fr. No. 6-7 in Fig . 1) and NA (Fr. No. 5-7 in Fig. 2A) fractions were prepared to compare the antigenicity among them. The results are shown in Table 3. The titers of anti whole virion antiserum in both HI and NI tests were lower when HA-NA fraction was used as an antigen than those obtained when whole virion or pre-eluted fraction was used as antigen (Table 3). On the other hand, the antiserum against HA-NA fraction reacted well with the whole virion and pre-eluted fraction (Table 3, lower column). These suggested that there was no significant difference between the antigenicities of the samples. The anti HA-NA antiserum, however, was found to have a relatively low NI titer for its HI titer (Table 3).

When the antigenic difference between the NA and the HA-NA fractions was checked by the double diffusion (Ouchterlony) method centering the anti NA antiserum, all the precipitation lines of each well fused together; no difference in the antigenicities between the NA samples was recognized (data not shown).

DISCUSSION

The purification of the HA protein is very important for the production of monoclonal antibodies against HA, which can be used to localize the antigenic deteminants on

HA molecules of each subtype, compare the primary structures of these determinants, and estimate the regions with HA activity or pH-dependent fusion activity. In this paper, we adopted fetuin-lignad affinity chromatography to purifiy the HA protein after the influenza virus was purified by sucrose gradient centrifugation and treated with 0.05% Tween 80-ether. In the USSR (H1N1) and Adachi (H2N2) strain, highly purified HA fraction could be obtained by affinity chromatography. In the Aichi strain (H3N2), however, the HA peak was separated into two portions after the sample was eluted by the elution buffer. The second peak may correspond to the highly purified HA peak from the USSR and Adachi strains, but the first peak showed a ratio 1: 1 of HA titer to NA titer. The latter underwent N- (p-aminophenyl) oxamic acid-ligand affinity chromatography for the removal of NA protein, which failed to separate the HA from the mixture of HA and NA proteins. This suggests that HA and NA combine inseparably with each other in H3N2 subtype resulting in causing steric inhibition in HI and NI tests.

The findings mentioned above may be connected with Arora's report (1980) which revealed the existence of protein molecules with both HA and NA activities in the Aichi strain or Laver's report (1984) about NA showing HA activity. We are now investigating what structure shows the intramolecular bond of HA and NA, if such a bond can be found, and whether the HA molecule may combine with the NA molecule by a strong intramolecular bond.

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