

Fluorometric Measurement of Neuraminidase Activity of Influenza Viruses^{*)}

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ABSTRACT

A rapid and simple assay method for determining neuraminidase activity was investigated fluorometrically with 4-methylumbelliferyl-N-Ac- α -D-neuraminide as a substrate. The time required for performance was less than 3 hr and the activity could be simply measured with a fluorescent spectrophotometer.

Neuraminidase activity in culture supernatant of MDCK cell infected with a small quantity of influenza A/Aichi/2/68 virus was demonstrated by the fluorometric assay 24 hr after infection, when no viral cytopathogenic effect could be observed and no viral multiplication could be demonstrated by the hemagglutination test and the colorimetric neuraminidase assay method using fetuin as a substrate.

The result was that the minimum number of virion detectable by the fluorometric neuraminidase assay method was approximately 10^3 order.

INTRODUCTION

Neuraminidase (sialidase) hydrolyzes substrates containing α -ketosidically linked N-acetylneuraminic acid (sialic acid). The assay methods for this enzyme have already been reported^{2,3)}.

Recently, Myers et al.⁵⁾ synthesized 4-methylumbelliferyl (4-MU)- α -ketoside of N-acetylneuraminic acid and developed a highly sensitive and simple assay method for neuraminidase using this 4-MU derivative as a fluorescent substrate. Yolken et al.¹¹⁾ suggested that this fluorometric neuraminidase assay method would be advantageous for detecting a small quantity of influenza virus not only from preparations of cultivated influenza virus but also from nasal wash specimens of human volunteers experimentally infected with the virus. Moreover, Shibuta et al.⁷⁾ reported that the fluorometric assay method could be used for detecting neuraminidase activity of two variants of parainfluenza virus type 3, which had been

shown to have no detectable neuraminidase activity, and referred to a correlation between syncytial formation and viral neuraminidase activity.

These pioneering works, however, were performed with 4-MU substrate synthesized by each worker himself. Now, the fluorescent substrate, 4-MU-N-Ac- α -D-neuraminide, has become commercially available.

In this paper, we describe the fluorometric assay method for determining viral neuraminidase and its availability for detecting a small number of influenza virus grown in experimental conditions.

MATERIALS AND METHODS

1. *Virus.*

Influenza viruses investigated in the present study were A/Aichi/2/68 (H3N2), A/USSR/92/77 (H1N1) and B/Kanagawa/3/76 (B). All strains were propagated in Madin-Darby canine kidney (MDCK) cells according to the method described by Tobita⁸⁾ and Tobita et al.⁹⁾ and

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the culture supernatant fluid incubated at 34°C for 3 or 4 days was used. The crude virus solution was stored at -80°C until use. Determination of infectivity (plaque forming unit: PFU) of these viruses was performed according to the method described by Tobita⁹⁾ and Tobita et al.⁹⁾, and hemagglutination titer was determined by the microtiter method.

2. Fluorometric neuraminidase assay method.

The method for enzyme activity described by Yolken et al.¹¹⁾ was somewhat modified. The substrate, 4-MU-N-Ac- α -D-neuraminide ammonium salt tetrahydrate (MW=556.52: Koch Light Laboratories), was dissolved in 2-methoxyethanol (methyl cellosolve: Wako Junyaku) to prepare 5 mM stock solution. The stock solution was stored at 4°C in a refrigerator. The working solution was prepared in the assay buffer, 0.1 M phosphate buffer (pH 5.8) containing 2 mM CaCl₂, to make the final concentration of the substrate of 0.1 mM.

A 100 μ l-aliquot of virus suspension was incubated with 100 μ l of the working solution at 37°C in a water bath. After incubation for 150 min, the reaction was terminated by adding 3.3 ml of 50 mM glycine buffer (pH 10.4) containing 5 mM EDTA, and the fluorescent intensity was measured at an excitation wavelength of 360 nm and an emission wavelength of 440 nm with a fluorescent spectrophotometer (Hitachi model 204).

β -Methylumbelliferone (Wako Junyaku) in 50 mM glycine buffer (pH 10.4) served as a standard. Enzyme activity was expressed as p moles of the substrate hydrolyzed/150 min/100 μ l of sample.

3. Colorimetric neuraminidase assay method.

Compared with the fluorometric assay method, the colorimetric determination of neuraminidase activity of influenza virus was performed according to the standard method described by Aymard-Henry et al.¹⁾ using fetuin (Type III: Sigma) as a substrate. N-Ac-neuraminic acid (Type VI: Sigma) in 0.1 M phosphate buffer (pH 5.9) containing 3 mM CaCl₂ served as a standard. Enzyme activity was expressed as p moles of N-Ac-neuraminic acid released/18 hr/50 μ l of sample.

RESULTS

1. Basic conditions for fluorometric neuraminidase assay method.

Basic conditions for fluorometric measurement of neuraminidase activity of influenza virus were investigated by using A/Aichi/2/68 strain.

The time course of viral neuraminidase activity was shown in Fig. 1.

When the reaction was terminated every 30 min after the incubation of the mixture of virus suspension and the substrate (0.1 mM) at 37°C, the viral neuraminidase activity increased linearly until 150 min later (solid circles). When the reaction was terminated every 60 min, the neuraminidase activity seemed to reach the plateau at 180 min-incubation (open circles). The incubation time was determined to be 150 min.

An optimal concentration of the substrate was investigated. Enzyme activity was almost proportional to concentrations of the substrate from 0.05 to 0.2 mM, but it tended to saturate at 0.1 mM (Fig. 2).

The effect of calcium ion on the viral neuraminidase activity was shown in Fig. 3. A/Aichi/2/68 strain showed high catalytic activity in the presence of CaCl₂ from 1 mM to 4 mM in 0.1 M phosphate buffer (pH 5.8), but a dramatic inhibition in the activity was demon-

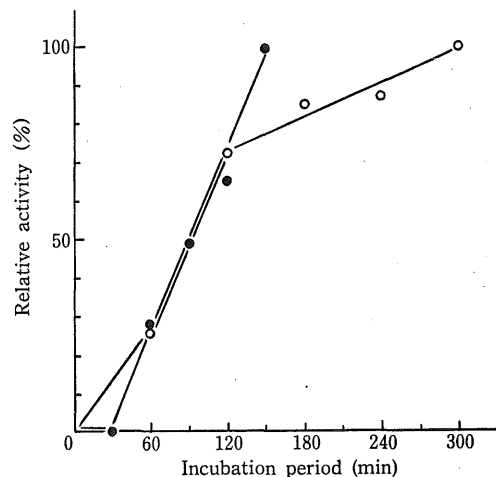


Fig. 1. Time course of neuraminidase activity of influenza A/Aichi/2/68 virus. A 100 μ l-aliquot of the suspension of influenza A/Aichi/2/68 virus was incubated at 37°C with an equal volume of the substrate (0.1 mM) and the viral neuraminidase activity was measured fluorometrically. Open circles represent the relative activity (%) measured every 30 min until 150 min and closed circles every 60 min until 300 min.

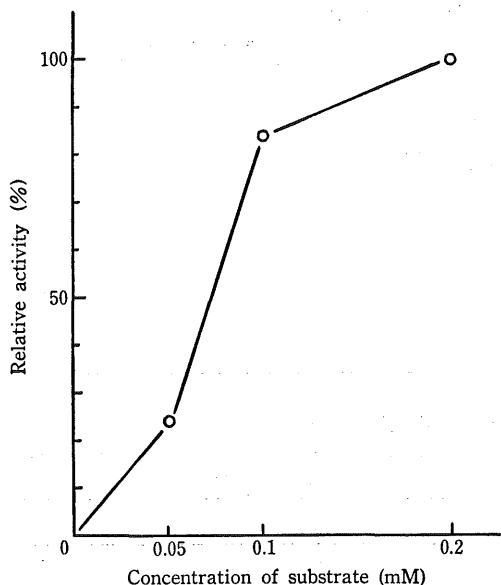


Fig. 2. Effect of concentration of substrate on neuraminidase activity of influenza A/Aichi/2/68 virus. Neuraminidase activity was determined 150 min after incubation at 37°C using 0.05 to 0.2 mM of 4-MU-N-Ac- α -D-neuraminide.

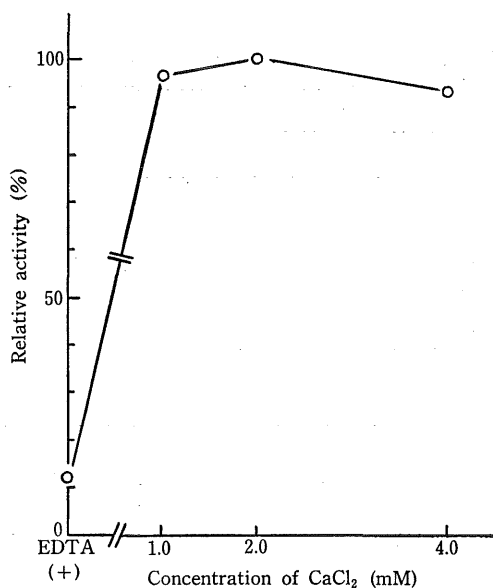


Fig. 3. Effect of concentration of CaCl₂ on neuraminidase activity of influenza A/Aichi/2/68 virus. Neuraminidase activity was determined 150 min after incubation at 37°C using 0.1 M phosphate buffer (pH 5.8) containing 1.0 to 4.0 mM of CaCl₂ as the assay buffer. Dependency of the enzyme activity on calcium ion was demonstrated using 0.1 M phosphate buffer (pH 5.8) containing 10 mM EDTA instead of CaCl₂ in the buffer.

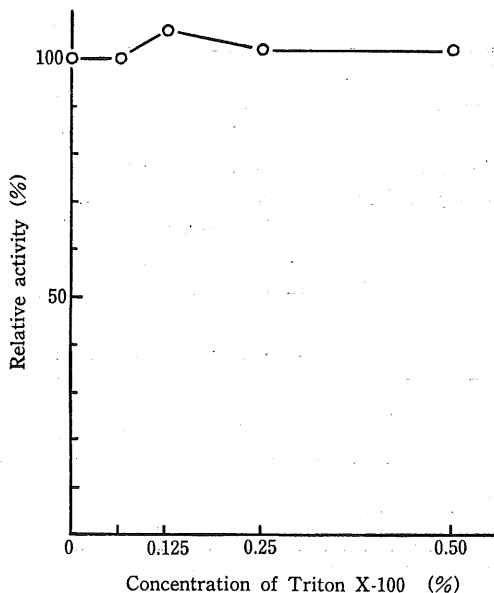


Fig. 4. Effect of Triton X-100 on neuraminidase activity of influenza A/Aichi/2/68 virus.

strated by the use of 0.1 M phosphate buffer (pH 5.8) containing 10 mM EDTA as an assay buffer (Fig. 3).

No effect of Triton X-100 on the viral neuraminidase activity was shown over the range from 0.1 to 0.5% (v/v) (Fig. 4).

From the results obtained, the condition of the fluorometric assay method for viral neuraminidase activity was settled as shown in Table 1.

2. Fluorometric measurement of neuraminidase activity of influenza virus.

Hemagglutination activity, infectivity (PFU/0.1 ml) and neuraminidase activity were measured on the culture supernatant fluid of each strain of influenza virus. The results are summarized in Table 2.

Every strain showed hemagglutination, plaque formation and neuraminidase activity. The catalytic rate of substrate was higher when the neuraminidase was measured by the colorimetric method using fetuin as a substrate than by the fluorometric method using 4-MU-N-Ac- α -D-neuraminide as a substrate, especially with B/Kanagawa/3/76 strain.

No neuraminidase activity was detected in the supernatant of non-infected MDCK cells treated with freezing and thawing or 0.1% Triton X-100.

Next, the minimum dose of virus detectable

Table 1. Fluorometric neuraminidase assay method

Enzyme solution	: Culture supernatant fluid of MDCK cell monolayer infected with influenza virus
Substrate	: 4-Methylumbelliferyl-N-Ac- α -D-neuraminide (Koch Light Laboratories, final concentration=0.1 mM)
Assay buffer	: 0.1 M phosphate buffer (pH 5.8) containing 2 mM CaCl ₂
Assay method	: Enzyme solution 100 μ l Buffered substrate 100 μ l —incubated at 37°C for 150 min —terminated by addition of 3.3 ml of 50 mM glycine buffer (pH 10.4) containing 5 mM EDTA —fluorescence is measured at E ₃₆₀ F _{440 nm}

Table 2. Viral neuraminidase activity of influenza virus

Influenza virus	HA titer	Infectivity (PFU/0.1 ml)	Neuraminidase activity*	
			(1)	(2)
A/Aichi/2/68 (H3N2)	256	2.60 $\times 10^7$	64,750 (25,900)	421,560 (46,840)
A/USSR/92/77 (H1N1)	256	2.93 $\times 10^7$	17,400 (6,960)	137,700 (15,300)
B/Kanagawa/3/76 (B)	128	3.67 $\times 10^7$	25,283 (10,113)	1,208,400 (134,267)

* Neuraminidase activity was expressed as p moles of 4-MU/150 min/100 μ l of sample with the fluorometric assay method using 4-MU-N-Ac- α -D-neuraminide as a substrate (1), and p moles of N-Ac-neuaminic acid/18 hr/50 μ l of sample with the colorimetric assay method using fetuin as a substrate (2). Parentheses show activities as p moles/hr/100 μ l of sample.

Table 3. Various activities of influenza A/Aichi/2/68 virus grown in monolayer of MDCK cell

Influenza virus	CPE	HA titer	Neuraminidase activity ¹⁾		Infectivity (PEU/0.1 ml)
			(1)	(2)	
A/Aichi/2/68					
12 hr	—	<4	7.3	0	2.89 $\times 10^4$
24	—	<4	185.5	0	1.54 $\times 10^5$
36	—	40	14,863	62,640	2.05 $\times 10^7$
48	(+) ²⁾	160	60,250	244,440	3.92 $\times 10^7$
Control					
48 hr	—	<4	5.5	0	0
A/Aichi/2/68					
original solution		256	71,000	421,920	2.60 $\times 10^7$
diluted to 1:2 $\times 10^7$		<4	5.0	0	1.30

1) (1): p moles/150 min/100 μ l of sample with fluorometric assay method

(2): p moles/18 hr/50 μ l of sample with colorimetric assay method

2) (+) shows that the cytopathogenic effect of influenza virus against MDCK cell monolayer is barely observed.

was investigated on MDCK cell monolayer grown in tissue culture dish (35 \times 10 mm: Falcon 3001, Becton Dickinson Labware) infected with a small quantity of influenza virus A/Aichi/2/68 strain (1 or 2 PFU/monolayer).

The appearance of viral cytopathogenic effect, hemagglutination titer, infectivity and neuraminidase activity were measured periodically. The results are shown in Table 3.

The neuraminidase activity was demonstrated

by the fluorometric method within 24 hr of incubation, but the colorimetric method and hemagglutination activity were detected 36 hr after incubation. The viral cytopathogenic effect was barely observed 48 hr after incubation. Judging from the number of virus particles, the minimum dose of virion required for neuraminidase assay was estimated to be approximately 10^8 order with the fluorometric assay method and 10^5 or more with the colorimetric assay method, respectively.

DISCUSSION

Neuraminidase is one of the proteins present in the envelope of most myxo- and paramyxovirus particles, and the activity increases markedly in tissues infected with these viruses *in vitro* or *in vivo*. These facts suggest that detection of viral neuraminidase activity in the culture supernatant fluid possibly demonstrates viral multiplication. However, neuraminidase assay method for detecting viral multiplication has not widely been applied due to its complexity of the procedure.

Tuppy and Palese¹⁰ synthesized a chromogenic substrate, 2-(3'-methoxyphenyl)-N-Ac- α -neuraminic acid, and succeeded in visualizing the presence of Newcastle disease virus or influenza virus in chick embryo fibroblast monolayer as the neuraminidase-active foci⁶. The results demonstrated that the method could successfully be used for the assay of myxo- and paramyxoviruses having neuraminidase with no cytopathogenic effect during the replication.

Recently, Yolken et al.¹¹ also demonstrated the neuraminidase activity in preparations of cultured cells as well as nasal specimens containing a small quantity of influenza viruses by a highly sensitive fluorometric neuraminidase assay method developed by Myers et al.⁵

In our present results, the minimum number of virion of influenza virus A/Aichi/2/68 strain for detecting neuraminidase activity was approximately 10^8 order, and the activity could easily be detected 24 hr after infection, when the viral multiplication could be demonstrated by neither the hemagglutination test nor the colorimetric neuraminidase assay method. As the number of influenza virus contained in nasal washings in the acute phase of illness comes up to 10^4 PFU/ml⁹, a direct demonstration of the presence of influenza virus in specimens may

possibly be achieved by the fluorometric neuraminidase assay method.

In our present study, a marked difference in catalytic rate was observed between the two methods for neuraminidase assay. The difference might be attributed to the substrate employed. Fetuin contains two different neuraminic acid derivatives, N-acetyl and N-glycolyl neuraminic acid, which are hydrolyzed at different rates by various neuraminidase⁴, while 4-MU-N-Ac- α -D-neuraminide is a homogenous product fully synthesized⁵.

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