Glycoconjugate J (1989) 6:219-227

Kinetic Studies on the Sialidase of Three Influenza B and Three Influenza A Virus Strains*

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Received January 31/April 14, 1989.

Key words: influenza B, influenza A, sialidase kinetics, neuraminidase

Sialidase of influenza virus type A has been extensively studied through structural and kinetic approaches. However, sialidase of influenza virus type B has been less investigated. In this work, we have studied the activity and some properties (optimal pH, K_M, V_{max}, thermal stability) of sialidase in three influenza virus strains of type B (circulating in the period 1983-86) and also the activity and properties of sialidase from three virus strains of type A circulating at the same period of time. The results show that the activity and the V_{max} was always higher for sialidase of type A viruses relative to those values of type B. Differences were also found for optimal pH and, in some cases, for thermal stability of the sialidase between strains belonging to the influenza viruses type A and B. However, the behaviour for the sialidase in all strains was very similar towards two competitive inhibitors. Thus, it could be suggested that the evolution pattern of the sialidase of both types of influenza viruses determines some modifications which result in a higher efficiency for sialidase of some strains of influenza virus type A, but maintaining in the two types of viruses a similar behaviour towards competitive inhibitors.

Some research on the variation of sialidase (neuraminidase, *N*-acylneuraminosyl glycohydrolase, EC 3.2.1.18) in the influenza A virus has been carried out using different approaches, such as the examination of the chemical structure of the glycoprotein, determination of its gene sequences, study of its antigenicity, and assays on the correlation between structural and antigenic changes (for reviews, see [1-6]).

The study of the active site pocket of the sialidase of virus type A [7, 8] showed that in three epidemic periods between 1957 and 1975 none of the variable regions around the active site pocket of this sialidase escaped without some alteration [4]. The eventual influence of

*Dedicated to Professor Maurice Leclerc by one of us (J.A.C.) on the occasion of his retirement.

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these changes on the sialidase activity has undergone only limited studies. In a previous paper [9], we have determined the sialidase activity and the kinetic parameters of human influenza A viruses belonging to three subtypes A (H1N1, H2N2 and H3N2) and found some differences. Unlike influenza A viruses, influenza B viruses do not undergo antigenic shift, as has been outlined by several authors [10, 11]. In addition, influenza B viruses do not seem to have a regular progression in the changes [12] and "drift" is probably not an accurate term for the antigenic variation in these viruses [5]. Nonetheless, the evolution pattern of influenza B viruses is slower than that of influenza A viruses [12], although few data are available for influenza B viruses [11, 12]. Oxford *et al.* [13] have outlined that the detailed biochemistry of the influenza B viruses is barely known in comparison with that of the influenza A viruses and that the evolution of these viruses is rather more complex than previously considered.

The aim of the present work is to extend our knowledge of the sialidase of three influenza B virus strains (circulating during the period 1983-86) and study the eventual influence of the minor changes ("drift") on this activity and its kinetic parameters. The sialidase activity and the same parameters of three influenza A virus strains (all H3N2) circulating at the same period of time were also determined.

Materials and Methods

Viruses

The virus strains used were A/Texas/85 (H3N2), A/Leningrad/83 (H3N2), A/Caen/85 (H3N2), B/URSS/83, B/URSS/85, and B/Ann Arbor/86. The viruses were isolated from humans and were grown and purified at the Pasteur Institute (Paris), as previously reported [14].

Chemicals

N-Acetylneuraminic acid and bovine serum albumin were obtained from Sigma Chemical Co (St. Louis, MO, USA). 2′-(4-Methylumbelliferyl)-α-*N*-acetyl-D-neuraminic acid (MU-NeuAc) was purchased from Kock-Light Laboratories (Colnbrook, UK). *N*-Acetylneuraminyllactose and lactose/galactose test-combination (catalogue No. 176303) were from Boehringer Mannheim (W. Germany).

Sialidase Assays

Enzyme activity was determined by the method of Warner and O'Brien [15] in 0.1 M potassium phosphate buffer, pH 5.9, with 1.5 mM MU-*N*-acetylneuraminic acid as substrate. The reaction mixture (50 µl) was incubated, the reaction stopped and the fluorescence measured as previously reported [16]. When the enzyme activity was assayed using *N*-acetylneuraminyllactose as substrate, the method described by Cabezas *et al.* [9, 14] was followed. This procedure is based on the fluorimetric determination of NADH produced in the coupled reactions.

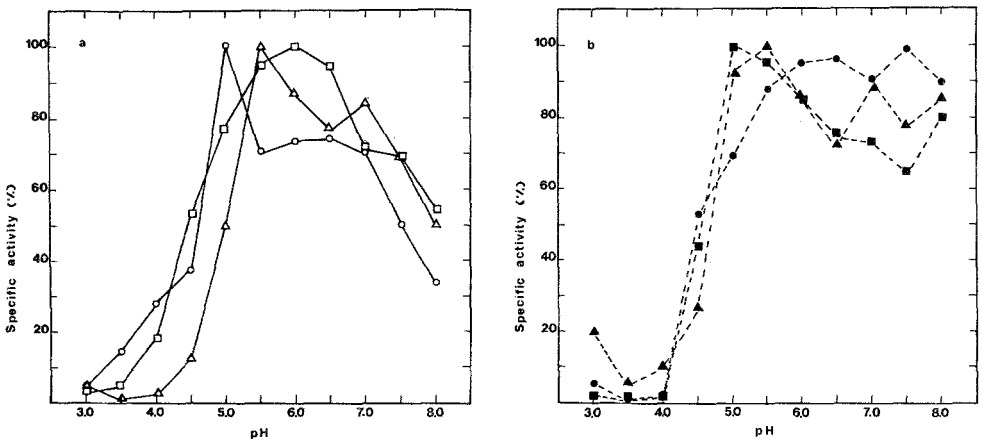


Figure 1. Effect of pH on sialidase activity from A (a) and B (b) influenza virus strains: A/Texas/85 (△), A/Leningrad/83 (○), A/Caen/85 (□), B/URSS/83 (●), B/URSS/85 (▲) and B/Ann Arbor/86 (■). 0.25 M potassium phosphate - citric acid buffer was used at pH values ranging between 3.0 and 6.0 and 0.25 M potassium phosphate buffer between 6.5 and 8.0.

One unit of enzyme activity (U) was defined as the amount of enzyme which hydrolyses 1 μmol of substrate/min under the assay conditions. Specific activities were expressed as U/mg viral protein. Protein was determined by the method of Lowry *et al.* [17] using bovine serum albumin as standard.

Determination of Kinetic Parameters

Michaelis constants (K_M) and maximal velocities (V_{max}) were determined by fitting the experimental data (in a Robust Weighting scheme) to the Michaelis-Menten equation with a non-linear regression least-squares fit computer program [18]. This computer program, marketed as "Enzfitter" by Elsevier Biosoft, uses the Marquardt algorithm as iterative technique. Tolerance in fitting was always achieved after two interactions, thus assessing the accuracy of our data. Each result is the mean of five assays. Inhibition constants (K_i) were determined from Dixon plots [19]

Haemagglutinin Activity.

The haemagglutinin titre for chicken red cells was determined using techniques already described [20], at the Institut Pasteur by one of us (C.H.).

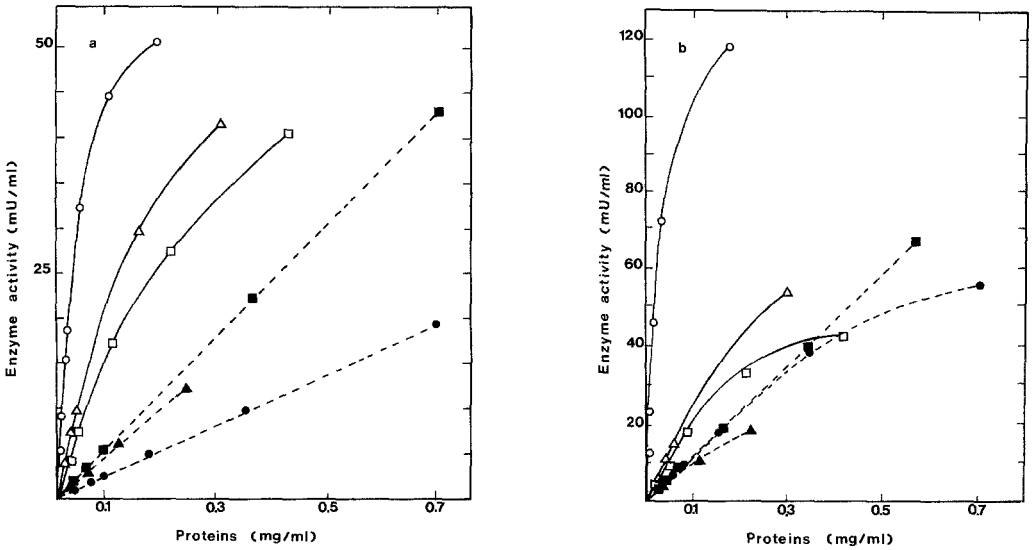


Figure 2. Variation of the sialidase reaction velocity on enzyme concentration, using MU-NeuAc (a) or *N*-acetylneuraminyllactose (b) as substrates. Virus strains used were: A/Texas/85 (△), A/Leningrad/83 (○), A/Caen/85 (□), B/URSS/83 (●), B/URSS/85 (▲) and B/Ann Arbor/86 (■).

Results

Optimal pH

As shown in Fig. 1, the optimal pH value was in the acidic range (5.0-6.5) for the sialidase from all the virus strains studied, using MU-NeuAc as substrate and the buffers 0.25 M potassium phosphate/citric acid (pH 3.0-6.0) and 0.25 M potassium phosphate (pH 6.5-8.0). Unexpectedly, a relatively high activity was observed around pH 8.0 only for the sialidase of type B strains.

Kinetic Studies

The variation of reaction velocity with enzyme concentration when using MU-NeuAc as substrate is shown in Fig. 2a and the corresponding results when *N*-acetylneuraminyllactose was used are shown in Fig. 2b. The type A virus strains showed higher activities than B strains on both substrates assayed.

The values of the apparent Michaelis constants (K_M) and maximal velocities (V_{max}) are shown in Table 1. When MU-NeuAc was employed as substrate, the lowest K_M values were obtained with the sialidase from type B viruses.

Table 1. Kinetic parameters of the sialidase on two substrates.

	MU-NeuAc[a]			*N*-acetylneuraminyllactose[a]		
	K_M (mM)	V_{max} (U/mg)	V_{max}/K_M K_M	V_{max} (mM)	V_{max}/K_M (U/mg)	
A/Texas/85	1.00	5.00	5.00	1.6	3.3	2.08
A/Leningrad/83	0.52	8.30	15.96	0.75	24.30	32.40
A/Caen/85	0.47	3.62	7.70	3.4	3.07	0.90
B/URSS/83	0.053	0.18	3.39	0.28	0.43	1.53
B/URSS/85	0.080	0.53	6.62	0.24	0.45	1.89
B/Ann Arbor/86	0.047	0.37	7.88	5.5	0.83	0.10

[a] The substrate concentrations used ranged from 0.1 to 1.5 mM for MU-NeuAc, and from 0.1 to 10 mM for *N*-acetylneuraminyllactose

The inhibition type was competitive for the virus sialidase assayed (data not shown) either with *N*-acetylneuraminic acid or 2,3-dehydro-2-deoxy-NeuAc as inhibitors. The 2,3-dehydro-2-deoxy-NeuAc produced a higher inhibition effect than *N*-acetylneuraminic acid (Table 2).

Thermal Stability

Two strains of type A, A/Leningrad/83 and A/Caen/85, were the most heat-stable. In contrast, the remaining studied strains showed a very low heat stability (Fig. 3).

Haemagglutinin Titre

The A/Leningrad/83 strain also showed the extreme values of haemagglutinin titre and protein concentration (Table 3) when compared with the other viruses assayed.

Discussion

To avoid modifications in the activity of the sialidase produced either by detergents or proteolytic cleavage agents when the enzyme is released from the viruses [16], we have used the whole purified influenza viruses as a source of sialidase. All virus strains were obtained and purified under the same conditions, and belong to virus types A and B circulating at the same period. They were also assayed under the same experimental conditions.

Table 2. K_i Values of the inhibitors studied.

	NeuAc[a] (mM)	2,3-dehydro-2-deoxy-NeuAc[a] (μM)
A/Texas/85	1.42	23.29
A/Leningrad/83	0.12	15.66
A/Caen/85	1.17	9.28
B/URSS/83	2	30
B/URSS/85	2.1	25
B/Ann Arbor/86	1.33	28.8

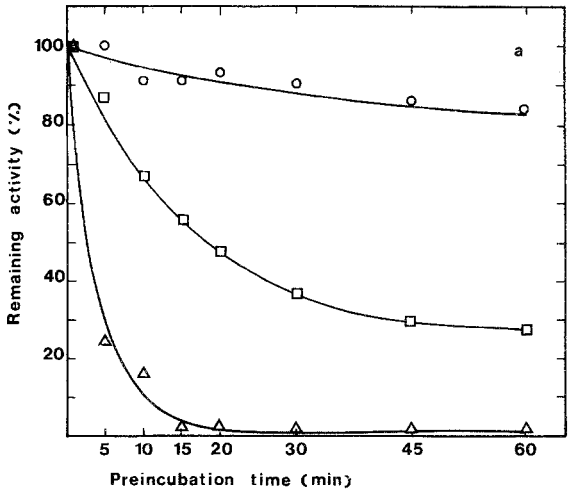
[a] The inhibitor concentrations ranged from 1 to 30 mM for *N*-acetylneuraminic acid, and from 25 to 100 μM for 2,3-dehydro-2-deoxy-NeuAc. The substrate used was the methylumbelliferyl glycoside at the same

Crystallization and preliminary X-ray analyses of the sialidase from two different influenza B virus strains have been recently reported [21]. Although the structure of the sialidase from influenza virus type B is not well known [11], the nucleotide sequence in the sialidase gene of some variant has been established; thus, the neuraminidase gene of B/Lee/40 shows some structural similarity, but also differences in comparison with two neuraminidase of type A, which are the N2 (A/RI/5/57) and the N1 (A/PR/8/34) [4]. Furthermore, it seems that the configuration of the active site pocket is the same in influenza B as in the A N2, in spite of the difference in amino-acid sequence [11]. These changes in the amino-acids of the active site could have an influence on the enzyme activity. We have found that sialidase of the influenza virus type B has a lower activity than that of type A. Perhaps the faster evolution pattern of the influenza viruses type A compared to that of type B, deduced from the comparison of the sequence divergence among genes of viruses belonging to both types [12], has favoured the appearance of a model of sialidase with a higher activity.

Parallel experiments were performed using either a natural substrate, *N*-acetylneuraminyl-lactose, or a synthetic compound, MU-NeuAc. Both substrates have been proven to be very convenient for this kind of assay [9, 14, 22].

In general, the kinetic parameters of type A and type B sialidase are significantly different. The characteristics (specific activity, K_M, V_{max}, K_i and thermal stability) of the sialidase of the A/Leningrad/83 strain show both the highest V_{max}/K_M ratio and the highest thermal stability for sialidases of virus influenza type A. Using MU-NeuAc as a substrate, the sialidase of type B strains exhibits a lower K_M, but also lower activity, and a lower V_{max} and V_{max}/K_M ratio than the corresponding values for the sialidase of type A.

The thermostability at 45°C was found to be higher for the sialidase of two strains of type A (one is A/Leningrad/83) with respect to the remaining assayed strains.



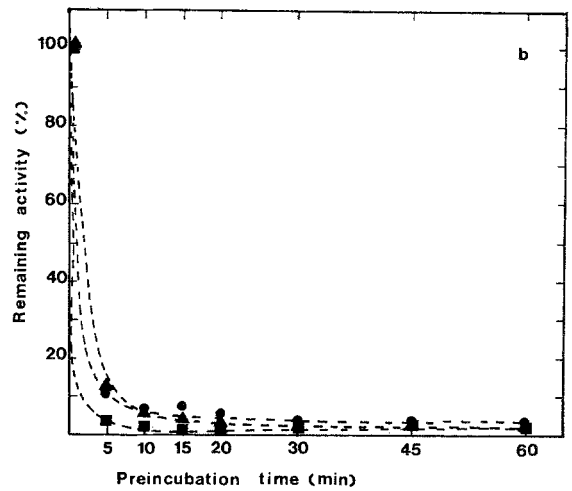


Figure 3. Thermal stability of the sialidase activity from two types of influenza virus strains, A (a) and B (b): A/Texas/85 (△), A/Leningrad/83 (○), A/Caen/85 (□), B/URSS/83 (●), B/URSS/85 (▲) and B/Ann Arbor/86 (■). Samples of the virus were heated in 80 mM potassium phosphate buffer, pH 5.9, at 45°C for different pre-incubation times. Remaining activity was determined using the methylumbelliferyl glycoside.

The behaviour of the sialidase of either type A or B viruses was similar for the inhibitors studied. However, the 2,3-dehydro-2-deoxy-NeuAc has a stronger inhibitory effect than *N*-acetylneuraminic acid. This result has also been observed for the sialidase of three subtypes A (H1N1, H2N2 and H3N2) of influenza viruses previously studied [9] and for that of a paramyxovirus, Newcastle Disease Virus [23].

Table 3. Haemagglutinin titre and protein concentration of the virus strains studied.

	HA	Protein (mg/ml)
A/Texas/85	1/5,000	0.59
A/Leningrad/83	1/40,000	0.35
A/Caen/85	1/6,000	0.85
B/URSS/83	1/5,000	1.40
B/URSS/85	1/6,000	0.45
B/Ann Arbor/86	1/32,000	1.43

Except for strain A/Leningrad/83, which exhibits the highest haemagglutinin titre in our assays, we have not found a correlation between sialidase activity and haemagglutinin titre.

Whilst influenza type A viruses cause the most severe diseases in epidemics and pandemics, influenza B viruses are believed to cause only epidemics; however, they circulate with those of type A. Thus, 78% of the influenza viruses isolated in the Northern half of France were type A and 22% type B, in the period of 1978-87, and in the five epidemic years of this period (1978, 1981, 1985, 1986 and 1987) the average of type B was 14% [24]. Furthermore, during the epidemic of the first months of 1988, the percentage of type B was significantly high in France (about 90%) (Hannoun, unpublished results).

Acknowledgements

This work was supported by a research grant from the Ministry of Education and Science of Spain (PR83-3019), and partly from the Integrated Action Nr. 56/226 between Spain and France. We are grateful to Mr. Henry Fitt for correcting this article and Miss Belén Blázquez for kindly typing the manuscript.

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