Biochemical Properties of Paramyxovirus
Duck/Mississippi/75 Neuraminidase

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SUMMARY

The neuraminidase activity of two strains of Duck/Mississippi/75 virus: DK/Mississippi/320 and DK/Mississippi/334 was studied. These neuraminidases hydrolyse the α2→3 and α2→8 ketosidic bonds of different substrates such as fetuin, N-acetyl neuramine lactose and colominic acid, but do not hydrolyse the α2→6 bonds of mucin type I and type II. The kinetic values of the neuraminidases, Michaelis constant, maximal and initial velocities and the effect of pH, temperature and detergents were also evaluated. The isolates differ mainly in the optimal pH and temperature conditions of activity. As with other paramyxovirus neuraminidases, the enzyme of DK/Mississippi/75 was destroyed by ionic but not non-ionic detergents.

Among the paramyxoviruses isolated from wild ducks by Webster et al. (1976), five were identified as a new avian paramyxovirus, distinct in their antigenic and biological properties (Kessler et al. 1979) and in their polypeptide composition (Kessler & Aymard, 1979). They were named Duck/Mississippi/75 (DK/Miss/75) viruses. The neuraminidase activity of DK/Miss/75 virus was easy to demonstrate in crude allantoic and amniotic fluids and was antigenically different from all known avian paramyxovirus neuraminidases but showed a slight cross-reaction with NDV neuraminidase (Kessler et al. 1979). In a previous study (Kessler et al. 1979), we observed slight differences in the neuraminidase activity of the different isolates related to the duck species from which the viruses were isolated, so we have chosen for the present study of the kinetic properties of DK/Miss/75 neuraminidase, two representative isolates, namely DK/Miss/320 and DK/Miss/334.

Both strains were grown in 8-day-old embryonated hen’s eggs; allantoic and amniotic fluids were harvested after 5 days’ incubation at 34 °C and then submitted to the purification procedure (Kessler & Aymard, 1979). Neuraminidase (EC 3.2.1.18) tests were performed as described previously (Kessler et al. 1979). Five substrates were tested: N-acetyl neuramine lactose (1 mg/ml; Sigma; 85 % α2→3 bonds and 15 % α2→6 bonds), fetuin (22 mg/ml) prepared as described by Han & Puck (1962), mucin I (5 mg/ml) from bovine submaxillary gland (Sigma; 5% of sialic acid), mucin II (5 mg/ml) from porcine stomach (Sigma; 1% of sialic acid) and colominic acid (5 mg/ml) from E. coli (Sigma). Neuraminidase activity was expressed in terms of nmol N-acetyl neuraminic acid released/min/mg protein. Virus protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

This new avian paramyxovirus possessed a high level of neuraminidase activity and the neuraminidase to haemagglutinin ratio was 0.2. This value is higher than those ratios reported for other avian paramyxoviruses such as NDV and Yucaipa viruses (Alexander, 1974; Kessler et al. 1979), 0121 strain (Collings et al. 1975) and Turkey/Wisconsin and parakeet/Netherlands/449/75 viruses (Alexander & Chettle, 1978).

The neuraminidase of both isolates hydrolysed the α2→3 bonds of fetuin and neuramine lactose and the α2→8 bonds of the colominic acid but did not cleave the α2→6 bonds of mucin I and II. This substrate specificity of the DK/Miss/75 neuraminidase was charac-
Table 1. Kinetic constant activity of the neuraminidases of two
*DK/Miss/75 isolates*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Virus</th>
<th>$K_m$ ($\times 10^{-8}$ M)</th>
<th>$V_{max}$ (nm/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuin</td>
<td>DK/Miss/320</td>
<td>1.2</td>
<td>2025</td>
</tr>
<tr>
<td></td>
<td>DK/Miss/334</td>
<td>1.37</td>
<td>1230</td>
</tr>
<tr>
<td>Neuramine lactose</td>
<td>DK/Miss/320</td>
<td>10.4</td>
<td>1550</td>
</tr>
<tr>
<td></td>
<td>DK/Miss/334</td>
<td>4.2</td>
<td>1160</td>
</tr>
</tbody>
</table>

* Each value represents the arithmetic mean of three assays in duplicate, under optimal conditions of pH and enzyme concentration.

The specific activities of these two neuraminidases were quite similar and after 18 h incubation at 37 °C, the amount of NANA released was about 300 nmol/min/mg from fetuin, 100 nmol/min/mg from neuramine lactose and 40 nmol/min/mg from colominic acid. Determinations of the Michaelis constants ($K_m$) of both DK/Miss/75 neuraminidases after 2 h incubation, so as to be in their initial velocity phase, using concentrations of fetuin ranging from 2 to 22 mg/ml and concentrations of neuramine lactose ranging from 0.25 to 4 mg/ml, were obtained from Lineweaver-Burk plots (Table 1). The apparent affinity of the enzyme for fetuin and neuramine lactose was low with respective $K_m$ values of approx. $10^{-8}$ and $10^{-2}$ M. These values are of the same order as those given for NDV (Alexander, 1974) and HA2 virus (Kessler et al. 1977) but tenfold higher than those given for NDV (Drzeniek et al. 1966; Brostrom et al. 1971) and for Sendai Yucaipa and mumps viruses (Brostrom et al. 1971). The maximum velocity of the enzyme did not differ from that of the other human and avian paramyxoviruses neuraminidases (Brostrom et al. 1971).

The specific activity of the DK/Miss/75 virus neuraminidase was shown to be three times higher using fetuin (300 nm/min/mg) than using neuramine lactose (100 nmol/min/mg) although this difference could be explained by the respective concentrations of the substrate; fetuin was used at a concentration of $K_m/2$ and neuramine lactose at a concentration of $K_m/6$.

The effects of pH on the neuraminidase activity of DK/Miss/320 and DK/Miss/334 isolates were established by using a citrate (0.1 M)–phosphate (0.2 M) buffer, with values ranging from 2.3 to 7.4 and fetuin or neuramine lactose as substrates. The reaction was allowed to proceed at 37 °C for 2 h in order to reach the initial velocity phase and for 18 h to measure the final effect on released NANA. The results (Fig. 1a) were as follows: (i) the activity–pH profile of each enzyme was the same when tested for 2 or 18 h; (ii) for each strain the pH optimum was lower for fetuin than neuramine lactose; for DK/Miss/320 and DK/Miss/334 neuraminidases the optimum pH were, respectively, 4.6 and 3.8 for fetuin and 6.2 and 5.4 for neuramine lactose. These results were in agreement with those reported for NDV by Drzeniek et al. (1966).

The release of NANA by DK/Miss/320 and DK/Miss/334 neuraminidases from neuramine lactose and fetuin substrates was studied after various incubation times at 37 °C and at the optimum pH. With fetuin as substrate, the initial velocities of DK/Miss/320 and DK/Miss/334 neuraminidases were respectively 880 and 710 nmol/min/mg and were constant for the first 4 h; by then the velocity had reached about 60% of the initial value and remained constant for a further 4 h after which it decreased to 0 by the 15th hour. For neuramine lactose, the initial velocities were, respectively, 215 and 290 nmol/min/mg and remained constant for 1 h; they then decreased in a similar way as for the fetuin substrate and reached
Fig. 1. (a) Effect of pH on DK/Miss/75 virus neuraminidase activity. Samples diluted to release a maximal amount of 50 nmol NANA were incubated with fetuin or N-acetyl neuramine lactose in citrate phosphate buffer for 18 h at 37 °C. The results are expressed as percentages of the enzyme activity at the optimum pH. Each value represents the arithmetic mean of three assays in duplicate. Incubated with neuramine lactose: △—△, DK/Miss/320; ■—■, DK/Miss/334; incubated with fetuin: ▲---▲, DK/Miss/320; □—□, DK/Miss/334. (b) Effect of pH on DK/Miss/320 virus bound and soluble neuraminidases. Samples diluted to release a maximal amount of 50 nmol NANA were incubated with fetuin or N-acetyl neuramine lactose in citrate phosphate buffer for 18 h at 37 °C. Results are expressed as percentage of the enzyme activity obtained at optimum pH: on neuramine lactose: ■—■, soluble enzyme; □—□, bound enzyme; on fetuin: ▲---▲, soluble enzyme; ○—○ bound enzyme.

0 by the 15th hour. When the results were expressed in term of percentage of total activity, the time course of the activity of both enzymes was similar for both substrates.

DK/Miss/75 isolates were clearly distinguishable from each other on the basis of their neuraminidase activity versus temperature profile. The enzyme activities of both isolates were tested after 2 h incubation, at temperatures ranging from 19 to 56 °C using samples diluted to release a maximum amount of 50 nmol NANA from neuramine lactose and from fetuin at their optimum pH. With fetuin, the temperature optima were 43 and 34 °C for DK/Miss/320 and DK/Miss/334 neuraminidases, respectively, and with neuramine lactose they were 34 and 40 °C, respectively. More than 50% of the activity was still detectable at temperatures ranging from 13 to 53 °C. Unlike that of HA2 virus (Kessler et al. 1977) the enzyme of DK/Miss/75 virus was active over a wide range of temperatures.

The temperature stability of the neuraminidases was calculated on the basis of two criteria; the first criterion, the temperature which inhibits 50% of enzyme activity after 5 min ex-
posure, was found to be 53 °C for both isolates. The DK/Miss/75 virus neuraminidase showed similar thermostability to other avian paramyxovirus neuraminidases such as NDV (Brostrom et al. 1971; Alexander, 1974), Bangor (Alexander, 1974), DK/Hong Kong/75 (Alexander et al. 1979), parakeet-Netherlands/448/75 (Alexander & Chettle, 1978) and 0121 virus (Collings et al. 1975). In contrast, HA2 virus (Kessler et al. 1977) and Sendai virus (Brostrom et al. 1971) have a thermolabile enzyme. The second criterion was the determination of the inactivation constant of the enzyme at different temperatures. DK/Miss/320 and DK/Miss/334 samples diluted to release about 50 nmol NANA after 18 h incubation at 37 °C were heated for 0 to 30 min at temperatures ranging from 37 to 60 °C and then chilled at 4 °C before being tested. The data collected during periods of exponential decline in activity are presented in terms of rate constant defined in the following equation for a first order inactivation: \[ 2.3 \log_{10} \left( \frac{N}{N_0} \right) = -kt \], where \( N_0 \) represents the original activity and \( N \) the activity at some time, \( t \), after heating. The inactivation constants were similar for DK/Miss/320 and DK/Miss/334 isolates with respective values of 0 min\(^{-1}\) at 37 to 40 and 45 °C, 0.025 and 0.022 min\(^{-1}\) at 49 °C, 0.096 and 0.106 min\(^{-1}\) at 52 °C, 0.260 and 0.283 min\(^{-1}\) at 56 °C and 1.281 and 1.281 min\(^{-1}\) at 60 °C. These values were similar to those of NDV (0.2 min\(^{-1}\) at 56 °C; Pierce & Haywood, 1973) and these results could provide an argument in favour of a phylogenetic relationship between NDV and DK/Miss/75 virus (Kessler et al. 1979).

Like other paramyxoviruses such as NDV (Inunuma et al. 1971) or HA2 virus (Kessler et al. 1977) the neuraminidase of DK/Miss/75 strains was destroyed to a large extent by ionic detergents (50% inactivation with SDS 0.1% or DOC 1%) but was not altered by non-ionic detergents such as Triton X-100 or Nonidet P40.

The glycoproteins of DK/Miss/75 virus were solubilized with Triton X-100 as previously described (Kessler & Aymard, 1979) and the bound and soluble enzymes were tested for pH effect and affinity for fettuin and neuramine lactose.

The optimum pH was found to be 1 unit lower for the soluble enzymes than for the bound ones (Fig. 1 b), but the \( K_m \) of the bound enzymes were identical to the soluble ones.

In conclusion, the neuraminidase activity of the DK/Miss/75 strain was characteristic of the paramyxovirus genus and particularly of the avian paramyxoviruses. The enzyme hydrolysed the \( \alpha 2----\alpha 3 \) and \( \alpha 2----\beta 8 \) bonds of substrates, its specific activity and thermostability were high and it was not inactivated by non-ionic detergents. The neuraminidases of both DK/Miss/320 and DK/Miss/334 were quite similar but could be distinguished by their optimum pH and optimum temperature for activity.

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