# PURIFICATION AND PROPERTIES OF TWO NEURAMINIDASES FROM *STREPTOCOCCUS VIRIDANS* II

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## 1. Introduction

*Streptococcus viridans* II belongs to the group of Gram-positive, α-hemolyzing cocci causing septicemia and endocarditis in man. Whereas occurrence and properties of neuraminidases (sialidase, acylneuraminosyl hydrolase, EC 3.2.1.18) in β-hemolyzing streptococci are well documented [1–5], little is known about these enzymes in viridescent strains [6,7]. Neuraminidases cleave α-ketosidic linkages between *N*-acylneuraminic acid (NeuAc) and glycoconjugates (i.e., oligosaccharides, glycolipids and glycoproteins) of vertebrate tissues and are therefore discussed as pathogenic factors in microbial infections [8]. They are also known to unmask antigenic sites of tumor tissues and are already used in cancer immunotherapy [9].

Neuraminidases of viral and bacterial origin show genus-dependent distinctions in their specificities towards different substrates and also in their affinities towards different linkage types of NeuAc, i.e., α2→3, α2→6, and α2→8, to the adjacent carbohydrate molecules [10]. Therefore, further knowledge on microbial neuraminidases may enable us to develop better tools for structure elucidation of glycoconjugates as well as for applications in living systems.

## 2. Material and methods

### 2.1. *Origin of substrates*

3′-Sialyllactose ($II^3$ NeuAc-Lac), 6′-sialyllactose, ($II^6$ NeuAc-Lac), disialyllactose ($II^3$-(←2αNeuAc8)$_2$-Lac), pentaose b ($III^6$ NeuAc-LcOse$_4$), pentaose c ($IV^6$ NeuAc-nLcOse$_4$) and disialyllacto-N-tetraose ($IV^3$ NeuAc,$III^6$ NeuAc-LcOse$_4$) were isolated from human milk and from cow's colostrum, respectively, as in [11]. Colominic acid was a generous gift of Dr Y. Uchida, Uji, Japan. Ganglioside $G_{M1}$ = $II^3$ NeuAc-GgOse$_4$ Cer and $II^3$ NeuAc-GgOse$_4$ were kindly provided by Dr H. Wiegandt, Marburg. Bovine brain ganglioside mixture and fetuin were purchased from Sigma Chemical Co., St Louis, MO, $\alpha_1$-acid glycoprotein (orosomucoid) from Behringwerke AG, Marburg, and bovine submaxillary mucin and NeuAcβ2→methylglycoside from Boehringer Mannheim GmbH. NeuAcα2→(3′-methoxyphenyl)-glycoside was a gift of NIH Resources Branch, Bethesda, MD. Human urinary glycoprotein was isolated as in [12].

### 2.2. *Preparation of enzymes*

*Streptococcus viridans* (Biotype II) strains were isolated from blood cultures of patients with sepsis and were cultivated and typed as in [7]. Cultures (24 h) (strain 011785) in tryptose phosphate bouillon were centrifuged at 100 000 × *g* for 30 min. The clear supernatants were dialyzed 3 times for 12 h against 0.03 M barbital-acetate buffer (pH 6.0) according to Michaelis [13]. The undialyzable residue was concentrated in an Amicon UM 10 ultrafiltration cell to 1/10th of its initial volume. The remaining solution was fractionated by gel filtration on a Sephadex G-100 column (90 × 2.6 cm) equilibrated with Michaelis buffer mentioned above. Neuraminidase activities of each column fraction were identified as below. Two neuraminidase peaks, a higher molecular weight neuraminidase I and a lower molecular weight neuraminidase II were identified and readily separated. The two enzymes were rechromatographed separately on a Sephadex G-100 column (90 × 2.6 cm) calibrated with standard proteins for molecular weight determination and further purification. Protein assays were performed by the method in [14]. Neuraminidase I and II were concentrated again and stored at 4°C.

### 2.3. *Neuraminidase assay*

NeuAc-containing substrates were dissolved in 0.03 M barbitalacetate buffer (pH 6.0) so as to give a total of 50 μg/100 μl (≙ 1.50 mM) glycosidically linked NeuAc in each test. Additional buffer and an appropriate amount of enzyme preparation (i.e., 10–100 μl) to 200 μl final vol. were added. Incubation times were from 15 min to 24 h at 37°C. Blanks contained all the ingredients except the enzyme. NeuAc released was determined by the periodic acid/thiobarbiturate method according to [15]. With NeuAcα2→(3′-methoxyphenyl)-glycoside as substrate, neuraminidase activity was tested according to [16].

## 3. Results and discussion

In contrast to other streptococcus strains, *S. viridans* shows constantly high neuraminidase activities even after multiple transfers in vitro. However, enzyme production can be stimulated by addition of *N*-acetylmannosamine to the culture medium in an optimal concentration of $10^{-3}$ M. The enzyme preparation shows a remarkable high temperature stability: The temperature optimum is at 40°C. After 30 min incubation time, activity is reduced to 90% at 45°C, to 50% at 48°C, to 10% at 52°C, and 5% at 56°C, respectively.

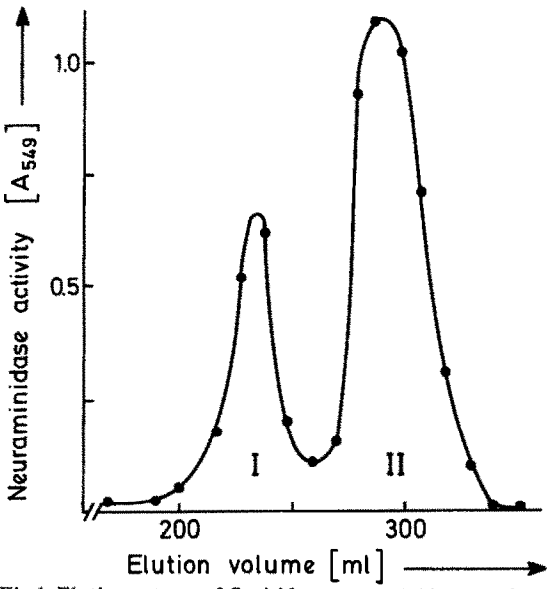


Fig.1. Elution pattern of *S. viridans* neuraminidases on Sephadex G-100. Column 90 × 2.6 cm, 0.03 M barbital-acetate buffer (pH 6.0). Determination of NeuAc released from II³NeuAc-Lac by the periodic acid/thiobarbiturate method according to [15]. Incubation, 14 h at 37°C.

As shown in fig.1, neuraminidase activity can be separated by gel filtration on Sephadex G-100 into two fractions, a higher molecular weight neuraminidase I ($M_r$ 86 000) and a lower molecular weight neura-

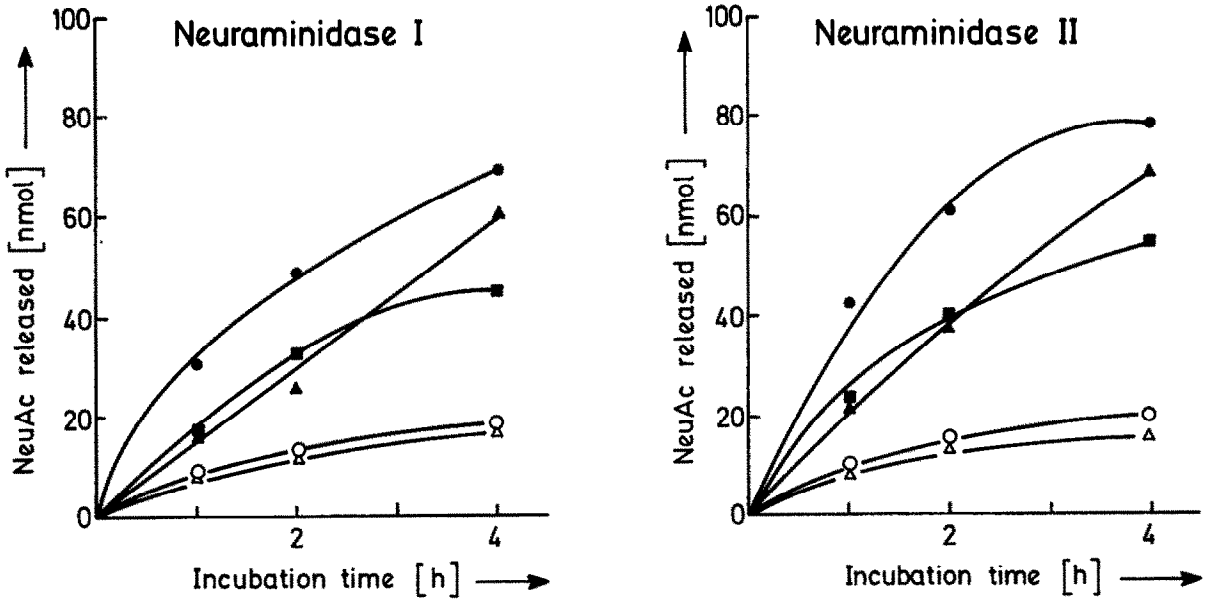


Fig.2. Release of NeuAc from different oligosaccharides by *S. viridans* neuraminidases I and II. Determination of NeuAc released as described. (●——●) II³NeuAc-Lac; (○——○) II⁶NeuAc-Lac; (▲——▲) II³(←2αNeuAc8)$_2$-Lac; (△——△) IV⁶NeuAc-nLcOse$_4$; (■——■) VI³NeuAc, III⁶NeuAc-LcOse$_4$.

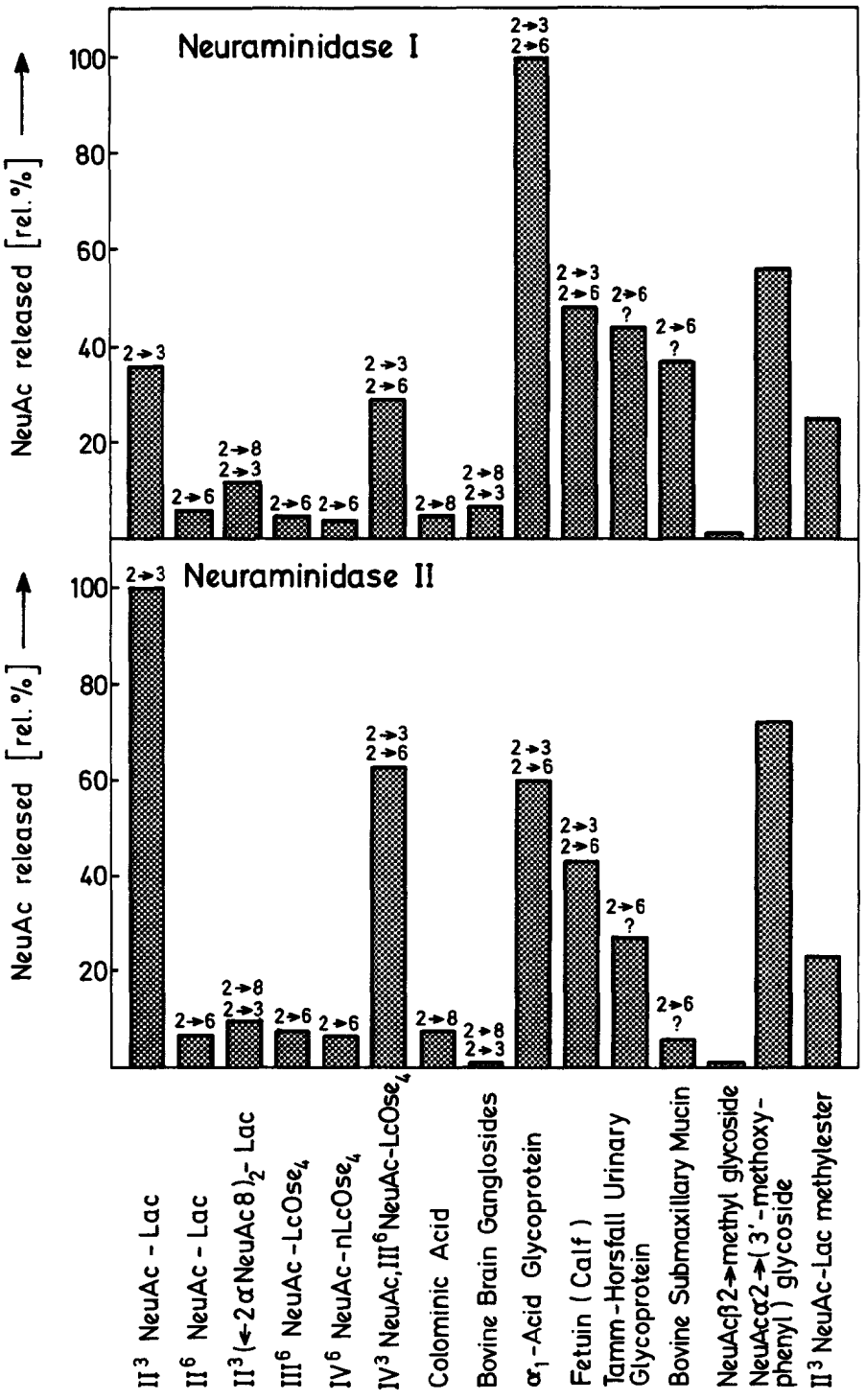


Fig. 3. Substrate specificity patterns of *S. viridans* neuraminidases I and II. The ordinate shows the relative percentage of cleavage rates, taking the substrate with the highest cleavage rate as 100. Substrate solutions contained ≐ 150 nmol glycosidically linked NeuAc. Enzymes were diluted to give linear cleavage rates up to 4 h for all substrates. Linkage types of NeuAc are given on top of the columns.

minidase II ($M_r$ 45 000). Considering the molecular weights, one could assume enzyme I is a dimer of enzyme II. However, as will be pointed out later, both enzymes show remarkable differences in their substrate type specificities. The pH optima are at 6.5 in veronal-acetate buffer for both enzymes. $Ca^{2+}$ increases the enzyme activities over $10^{-2}$–$10^{-5}$M. An inhibition by EDTA up to 50% was observed at $5 \times 10^{-2}$ M.

Fig.2 shows the cleavage velocities towards NeuAc-oligosaccharides. Among the different linkage types of NeuAc, both enzymes cleave the α2→3 linkage in $II^3$NeuAc-Lac preferentially, followed by the α2→8 linkage in $II^3$ (←2αNeuAc8)$_2$-Lac. The activities towards the α2→6 linkages in $II^6$NeuAc-Lac and in $IV^6$NeuAc-nLcOse$_4$ are remarkably low. Despite of numerous α2→8 linkages, colominic acid and bovine brain gangliosides are also little attacked by both enzymes under conditions used in the test. However, in contrast to ganglioside $G_{M1}$, the free oligosaccharide $II^3$NeuAc-GgOse$_4$ is cleaved to an extent of 20% compared with $II^3$NeuAc-Lac by enzyme II, indicating that the lipid part is mainly responsible for the resistance of $G_{M1}$ towards neuraminidase actions.

Fig.3 shows the specificity patterns of *S. viridans* neuraminidase I and II towards the different types of substrates under comparable conditions: The higher molecular weight enzyme I cleaves globular glycoprotein substrates, i.e., human $\alpha_1$-acid glycoprotein (orosomucoid) and calf fetuin, preferentially, followed by mucous substrates like Tamm-Horsfall urinary glycoprotein and bovine submaxillary mucin. In contrast, enzyme II prefers oligosaccharide substrates containing α2→3 linkages, whereas the activity towards glycoprotein substrates is considerably lower. Mucins are hydrolysed very slowly. Synthetic or semi-synthetic substrates like NeuAcα2→benzylketoside, NeuAcα2→(3′-methoxyphenyl)-ketoside or $II^3$NeuAc-Lac methyl ester are fairly good substrates for both enzymes, whereas NeuAcβ2→methyl-ketoside is not cleaved at all, demonstrating that *S. viridans* neuraminidases are α-glycosidases like all other neuraminidases known so far.

In general, the specificity patterns of both enzymes are similar to neuraminidases found in bacteria of low or mean pathogenicity towards man, i.e., *Clostridium septicum* [17], *Erysipelothrix rhusiopathiae* [18] and others [19]. The remarkable affinity of enzyme I towards α2→3 linked NeuAc compared with α2→6 linkages may be useful for the elucidation of NeuAc-linkages in glycoproteins as well as for specific alterations of cell membrane glycoconjugates.

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