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Purification and Kinetic Properties of Sialidase from *Clostridium perfringens*

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Summary: *Clostridium perfringens* sialidase was isolated from a culture medium of bacterial cells by ammonium sulfate precipitation (42–85%), followed by purification through Sephadex G-75 gel chromatography, DEAE A-50 anion exchange chromatography, FPLC medium pressure anion exchange chromatography and finally FPLC medium pressure isochromatofocussing. From 9 *l* culture medium 1.17 mg sialidase was isolated with a specific activity of 295 U/mg. The enzyme appeared to be homogeneous by analytical polyacrylamide gel electrophoresis. The molecular mass was measured to be 66 kDa. K_m values ranging from 0.6 to 1.6mM were determined for several oligosaccharides as substrates. The enzyme catalyzed transglycosylation reactions with methanol as a nucleophilic reagent competitive with water. In the enzymatic hydrolysis of the (3′-methoxyphenyl)glycoside of α-*N*-acetylneuraminic acid, increase of methanol concentration had no effect on the release of 3-methoxyphenol. This finding suggests that the formation of the enzyme-glycon intermediate is the rate-determining step for this substrate.

Reinigung und kinetische Eigenschaften der Sialidase aus Clostridium perfringens

Zusammenfassung: *Clostridium-perfringens*-Sialidase wurde aus einer Bakterien-Zellkultur isoliert. Die Reinigung erfolgte über Ammoniumsulfat-Fällung (42–85%), anschließende Chromatographie an Sephadex G-75, Anionenaustauscher-Chromatographie an DEAE A-50 sowie mit Hilfe von Mitteldruck-FPLC und schließlich Mitteldruck-FPLC-Isochromatofocussierung. Aus 9 *l* Kulturmedium wurden 1.17 mg Sialidase erhalten mit einer spezifischen Aktivität von 295 U/mg. Das Enzym verhielt sich in der analytischen Polyacrylamid-Gelelektrophorese homogen. Die molekulare Masse wurde zu 66 kDa bestimmt und die K_m-Werte für mehrere Oligosaccharide als Substrate lagen zwischen 0.6 und 1.6mM. Das Enzym katalysiert Transglycosylierungsreaktionen, wobei sich Methanol als nucleophiles Reagens kompetitiv gegenüber Wasser verhält. Bei der enzymatischen Hydrolyse des (3′-Methoxyphenyl)glycosids von α-*N*-Acetylneuraminsäure hatte ein Anstieg der Methanolkonzentration keine Wirkung auf die Freisetzung von 3-Methoxyphenol. Dieser Befund läßt vermuten, daß für dieses Substrat die Bildung des Enzym-Glycon-Zwischenproduktes der geschwindigkeitsbestimmende Schritt ist.

Key words: Neuraminidase, fast protein liquid chromatography, kinetics.

Enzymes:
N-Acetylneuraminate lyase, *N*-acetylneuraminate pyruvate-lyase (EC 4.1.3.3), also named *N*-acetylneuraminic acid aldolase; α-Galactosidase, α-D galactoside galactohydrolase (EC 3.2.1.22); α-Mannosidase, α-D-mannoside mannohydrolase (EC 3.2.1.24); Neuraminidase, acylneuraminyl hydrolase (EC 3.2.1.18), also named sialidase.

Abbreviations:
SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; MPN, 3-*O*-(3-methoxyphenyl)neuraminic acid; MNA, 3-*O*-(3-methoxyphenyl)-α-*N*-acetylneuraminic acid; HVL, haemine viande levure; RCA, reinforced clostridio agar.

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Sialidases (EC 3.2.1.18) are widely distributed in eukaryotic and prokaryotic cells[1]. They are exoglycosidases removing α-linked sialic acids from various substrates. Although sialidases from bacterial origin are frequently used for structure determination in glycoprotein research, relatively little is known about the mechanism of action. Recently the preferred rate of hydrolysis of sialyl linkages was studied[2]. The use of modified glycosides as substrates for sialidases has given information on the essential requirements for hydrolysis of substrates by these enzymes[3–5]. Recent experiments with 2-deoxy-2,3-dehydro-Neu5Ac have given some insight into the nature of the transition state[6]. For the enzyme several purification methods have been published, including affinity chromatography[7–9], preparative polyacrylamide gel electrophoresis[10], and others[11,12]. Here, we describe a new purification method for the sialidase of *Clostridium perfringens*, based on methods developed by Nees et al.[10], but using FPLC medium pressure chromatography as ultimate purification step. Of the purified enzyme K_m and V_{max} were determined for several substrates and rate equations in the hydrolysis were derived.

Materials and Methods

Materials

Reagents were of analytical grade: Todd Hewitt broth (Difco Laboratories), HVL medium[13], RCA (Oxoid), methanol, ammonium sulfate, potassium and sodium phosphate (Merck), imidazole (Fluka), bis-Tris and 3-*O*-(3-methoxyphenyl)-*N*-acetyl-D-neuraminic acid (Sigma), polybuffer 74, Sephadex G-75 and DEAE Sephadex A-50 (Pharmacia Fine Chem.), *N*-acetylneuraminyllactose (Boehringer), *N*-acetylneuraminic acid (Kantoishi Pharmaceutical Co., Ltd.). Purified Neu5Acα(2-3)- and Neu5Acα(2-6)-lactose were gifts from *Dr. G. Strecker*, and Neu5Acα(2-6)galactose was a gift from *Dr. D. J. M. van der Vleugel*.

Assay methods

Sialidase was assayed in a standard mixture containing 50 *μl* Neu5Acα(2-3)Lac (2 mg/m*l*) in 0.1M sodium/potassium phosphate buffer, pH 5.4, and 50 *μl* enzyme solution, at 37 °C. One unit (U) of sialidase activity is defined as the amount of enzyme that releases 1 μmol Neu5Ac from an excess of Neu5AcLac per minute at pH 5.4 and 37 °C. For the kinetic measurements sialidase was added in amounts up to 0.005 U. Incubation time varied between 20 and 180 min. The liberated Neu5Ac was determined by the thiobarbituric acid method of Warren[14] as described by Schauer[15]. Sialidase preparations were tested for the presence of the following enzymes: *N*-acetylneuraminate pyruvate-lyase with Neu5Ac[7], α-galactosidase with α-phenylgalactoside[16], mannosidase with 4-methylumbelliferyl mannoside[17] and protease with caseine[18] as substrates. Protein content was determined by the Lowry method[19] with bovine serum albumin as a standard. All spectrophotometric measurements were performed in a Cary 118 C. Carbohydrate analysis was carried out by subjecting samples to methanolysis, followed by gasliquid chromatography of the trimethylsilylated (*N*-reacetylated) samples on a capillary CPsil 5WCOT fused silica column (0.34 mm × 25 m, Chrompack, Middelburg, the Netherlands)[20].

Gel electrophoresis

Analytical polyacrylamide gelelectrophoresis (PAGE) was performed with samples containing 30 μg of protein at pH 8.9 in 7.5% gels and stained for protein with a 1% solution of Amido Black 10B (Serva)[21], or with silvernitrate by equilibrating the gel in a silvernitrate solution (1.9 g/*l*), followed by reduction with $NaHB_4$[22]. Sialidase activity was detected by incubating the gels in solutions containing 2-*O*-(3-methoxyphenyl)-α-*N*-acetylneuraminic acid (MNA) as a substrate[23]. PAGE was performed as described by Weber and Osborne[24]. Molecular mass markers were: soybean lipoxygenase I (M_r 100000), bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), α-chymotrypsinogen (M_r 25700), myoglobin (M_r 17800) and cytochrome *c* (M_r 11700).

Culture conditions

Clostridium perfringens ATCC 3626 was cultivated under anaerobic conditions at 37 °C. Freeze-dried cells were cultivated in 10 m*l* sterilized HVL medium and after 24 h of growth inoculated on RCA bloodagar plates. After 48 h this culture was controled for homogeneity by microscope, inoculated in 10 m*l* of fresh HVL medium and grown overnight (24 h). This culture was inoculated in 1 *l* of sterilized Todd Hewitt broth (40 g/*l*), and grown for 8 h. Growth rates were calculated by monitoring the turbidity at 1 h intervals with a Klett colorimeter[25].

Purification procedure

All purification steps were carried out at 4 °C except FPLC medium pressure chromatography, that was carried out at room temperature.

From 9 *l* culture medium the cellular material was removed by centrifugation at 10000 × *g* for 10 min. Ammonium sulfate was added in a concentration of 257 g/*l* (42% saturation), the mixture was stirred for 15 min and centrifuged at 18000 × *g* for 30 min. Then to the supernatant ammonium sulfate was added to a final concentration of 85% (562 g/*l*) to precipitate sialidase. The mixture was stirred for 30 min and centrifuged at 18000 × *g* for 30 min. The pellet was dissolved in 400 m*l* bidistilled water and dialysed for 48 h against 0.05M potassium phosphate buffer, pH 6.7. This sialidase solution was concentrated by the Amicon 8400 ultrafiltration system (Amicon, Massachusetts, U.S.A.) with an PM 30 membrane, then applied in three fractions to a Sephadex G-75 column (5 × 90 cm) and eluted with 0.25M potassium-phosphate buffer, pH 6.7. Sialidase-positive fractions were pooled, dialysed for 48 h against 0.1M imidazole/HCl buffer, pH 6.7, and applied to a DEAE A-50 anion exchange column (5 × 45 cm), equilibrated with 0.1M imidazole/HCl buffer, pH 6.7. The column was washed, and the enzyme was eluted

with a linear gradient of NaCl (0–300mM) in 0.1M imidazole/HCl buffer, pH 6.7, at a rate of 36 ml/h. Sialidase-positive fractions were pooled, dialysed for 48 h against 0.05M sodium/potassium-phosphate buffer, pH 6.7, and concentrated to 5 ml by ultrafiltration. This sialidase solution was divided into two equal portions, A1 and A2. Fraction A1 was dialysed against 0.1M imidazole/HCl buffer, pH 6.7, concentrated by ultrafiltration, applied to a HR 5/5 MonoQ high pressure anion exchange column, equilibrated with 0.1M imidazole/HCl, pH 6.7, and eluted with a non-linear gradient of 0–300mM NaCl. Sialidase-positive fractions were dialysed against 0.025M bis-Tris/HCl, pH 6.3, concentrated by ultrafiltration, and applied to a HR 5/20 MonoP high pressure isochromatofocusing column, equilibrated with 0.025M bis-Tris/HCl buffer, pH 6.3. Sialidase-positive fractions were dialysed against sodium/potassium-phosphate buffer, pH 6.7, and concentrated by ultrafiltration. The A2 fraction was applied to the MonoP column after dialysis against 0.025M bis-Tris/HCl buffer, pH 6.3, and concentration by ultrafiltration.

Kinetics

V_0, K_m and V_{max} were determined in the range of 0–3mM substrate.

Results

Growth of Clostridium perfringens

Final culture medium of *Clostridium perfringens* was a sterilized Todd-Hewitt broth (40 g/l), rich of sialic acid-containing glycoproteins. For optimal induction of sialidase production and excretion free Neu5Ac was added in a concentration of 0.3 g/l[26]. For 9 l culture medium the optimal growth period was 8 h, after which in total 774 U of sialidase activity were measured.

Purification of sialidase from culture medium

First 9 l culture medium was subjected to ammonium sulfate precipitation in 3 portions of

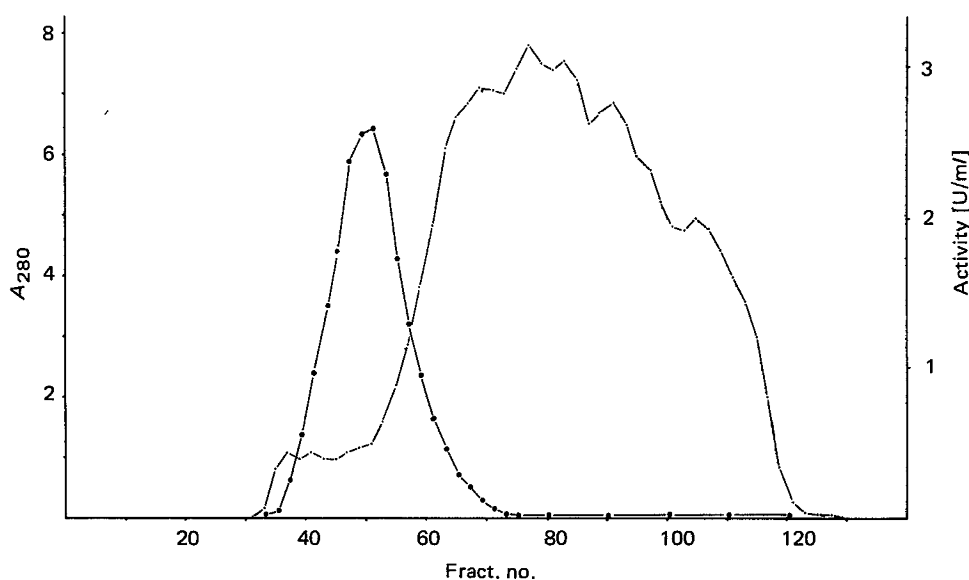


Fig. 1. Elution pattern on Sephadex G-75.
(—·—·—) Absorption at 280 nm; (●—●—●) sialidase activity; flow 46.2 ml/h; fraction size 15.1 ml.

Table 1. Purification of *Clostridium perfringens* sialidase.

	Total act. [U]	Total amount of protein [mg]	Spec. act. [U/mg]	Recovery [%]
Culture medium	774	26171	0.0296	100
$(NH_4)_2SO_4$ precipitation (42–85%)	681	4515	0.1508	88
Sephadex G-75	558	24.1	23.15	82
Sephadex A-50	502	4.2	119.5	90
Fraction A1	251	2.1	119.5	
MonoQ	216	0.92	234.8	86
MonoP	162	0.55	294.6	75
Fraction A2	251	2.1	119.5	
MonoP	183	0.62	296.2	73

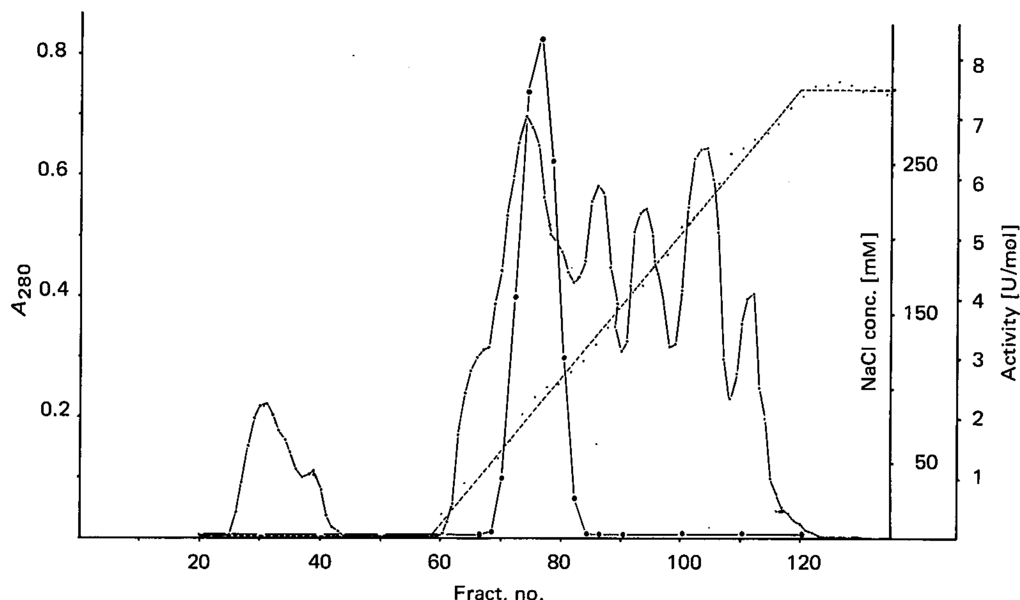


Fig. 2. Elution pattern on DEAE A-50.

(—·—·—) Absorption at 280 nm; (●—●—●) sialidase activity; flow 38 m*l*/h; fraction size 10.4 m*l*.

3 *l* each. After dissolving the precipitated sialidase preparation in bidistilled water, dialysis against 0.05M potassium-phosphate buffer and concentration by ultrafiltration, the enzyme solution was applied to a Sephadex G-75 column in three portions. The G-75 elution pattern is shown in Fig. 1. The 17-fold increase of the specific activity from 0.1508 to 2.596 U/mg protein (Table 1) was largely due to retardation of the low molecular mass constituents from the complex peptide broth used for cultivation. Fractions, containing sialidase, were pooled, and applied to a DEAE A-50 column. The material was eluted with a linear gradient of NaCl, as shown in Fig. 2, and divided into two equal portions A1 and A2.

Next the FPLC medium pressure chromatography system was used[27]. Fraction A1 was applied to the MonoQ column (Fig. 3) and the obtained sialidase-positive fraction was further fractionated on a MonoP column (Fig. 4). Fraction A2 was directly subjected to chromatography on a MonoP column (Fig. 5). From these chromatography patterns it was concluded that complete purification of the sialidase preparation was only possible on MonoP. Sialidase was eluted from the MonoP column at pH 4.7, as a homogeneous fraction of which the isoelectric point should be quite near this pH value. The effects of these purification steps on the specific activity of the enzyme preparations are shown by Table 1. Because of the short separation times we decided to run the FPLC system at room temperature. However, this temperature could be a factor responsible for the decrease in activity of the enzyme preparation during the MonoP purification step. On the MonoQ column the activity was better preserved, due to shorter separation times.

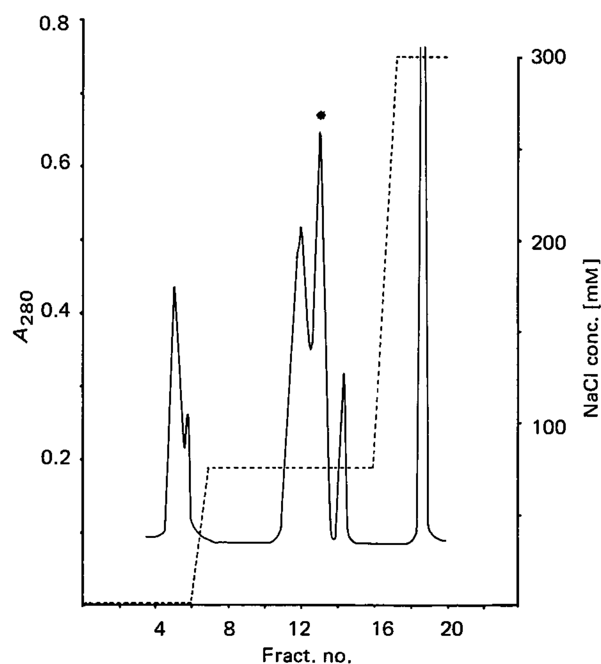


Fig. 3. Elution pattern of fraction A1 on MonoQ.

(——) Absorption at 280 nm; (– – –) NaCl concentration; flow 1.5 m*l*/min; fraction size 1 m*l*; x: sialidase-positive fraction.

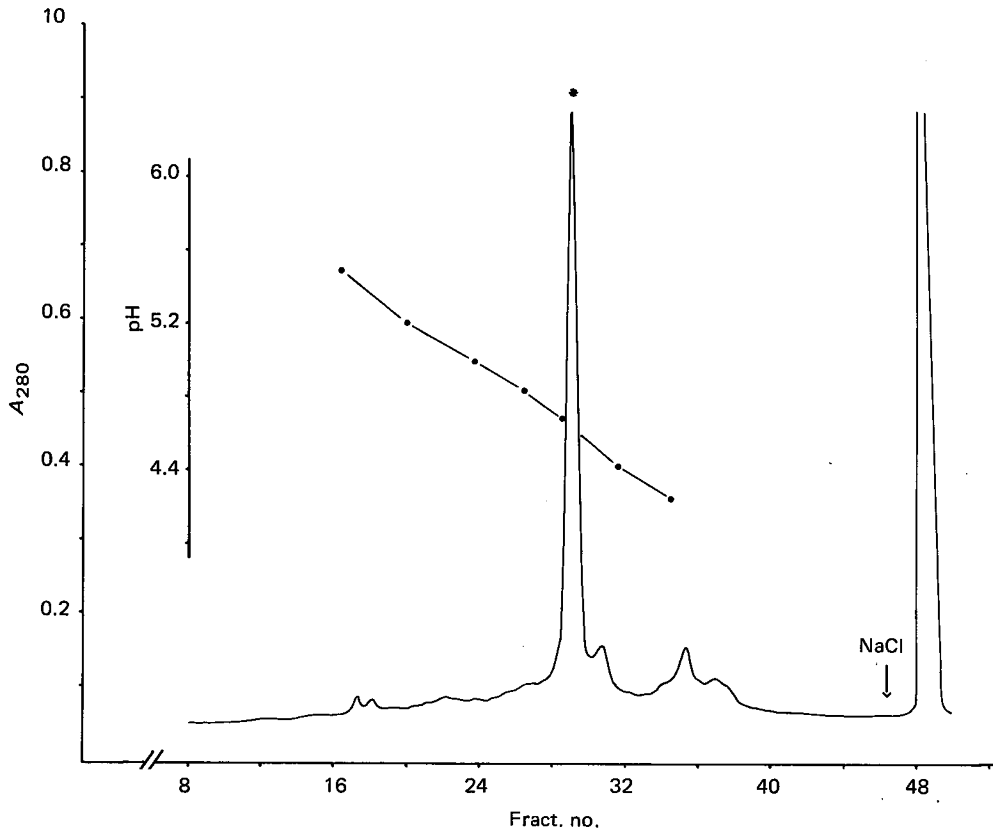


Fig. 4. Elution pattern on MonoP of the sialidase-positive fraction (A1Q) from MonoQ.
(——) Absorption at 280 nm; (●–●–●) pH; flow 0.5 m*l*/min; fraction size 1 m*l*; * : sialidase-positive fraction.

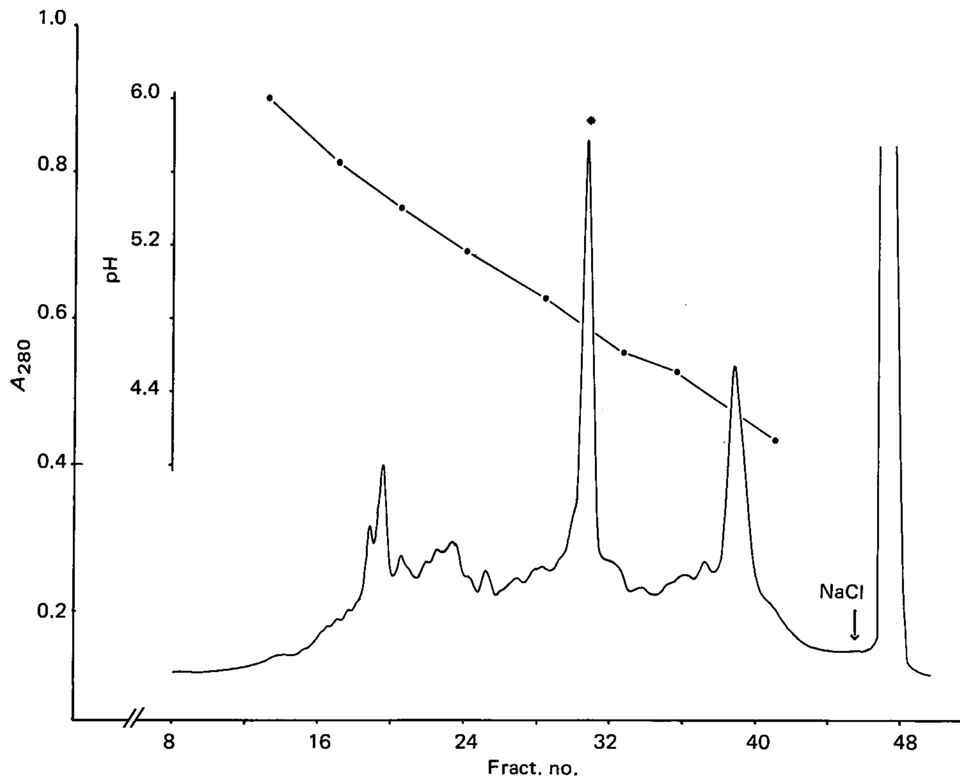


Fig. 5. Elution pattern of fraction A2 on MonoP.
(——) Absorption at 280 nm; (●–●–●) pH; flow 0.5 m*l*/min; fraction size 1 m*l*; *: sialidase-positive fraction.

Enzyme purity

The sialidase positive fractions from the HR 5/20 MonoP column were pooled, dialysed against 0.05M potassium-phosphate buffer, concentrated by ultrafiltration and analysed by PAGE. Both, Amido black and silver staining revealed only one protein band.

Molecular mass

The molecular mass of the enzyme as determined by SDS-PAGE and by filtration over a calibrated Sephadex G-100 column was found to be 67 000 and 65 000 Da, respectively. The calibration curve of the SDS-PAGE is shown in Fig. 6.

Sugar analysis

Sugar analysis of the pure neuraminidase samples revealed that *Clostridium perfringens* neuraminidase is not a glycoprotein, in contrast to *Streptococcus pneumonia* neuraminidase[28].

Kinetics

With Neu5Acα(2-3)Lac as substrate, the initial velocity was determined by regression analysis. At the optimum pH of 5.4 the value was 3.7 μmol Neu5Ac.

The purified enzyme showed typical Michaelis-Menten kinetics. Table 2 shows the apparent K_m and V_{max} for several substrates. A decrease in size of the aglycon leads to a decrease in K_m.

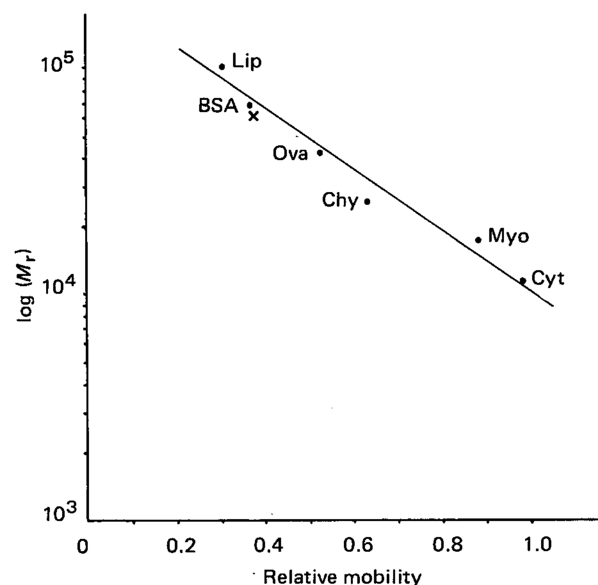


Fig. 6. Determination of the molecular mass of sialidase from *Clostridium perfringens* with SDS-PAGE:

x: Sialidase, Lip, soybean lipoxygenase; BSA, bovine serum albumin; Ova, ovalbumin; Chy, α-chymotrypsinogen; Myo, myoglobin; Cyt, cytochrome *c*.

Table 2. Apparent K_m values for several substrates determined for *Clostridium perfringens* sialidase.

Substrate	K_m [mM]	V_{max}
Neu5Acα(2-3)-lactose	1.6	3.7
Neu5Acα(2-6)-lactose	1.2	1.2
Neu5Acα(2-6)-galactose	0.8	0.7
MPN	0.6	0.6

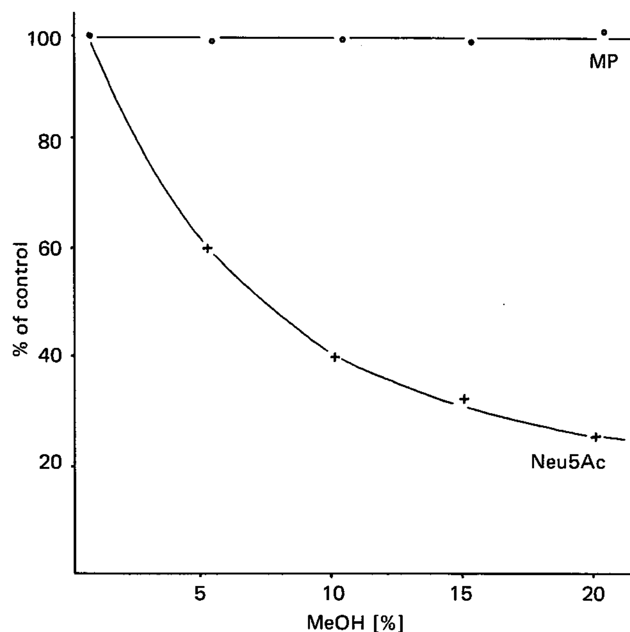
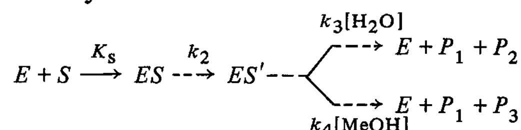


Fig. 7. Effect of methanol on the hydrolysis of MPN by *Clostridium perfringens* sialidase.

MP, 3-methoxyphenol.

The rate of hydrolysis of Neu5Acα(2-3)-lactose is about three times higher than that of the α(2-6) isomer, in agreement with results published by Michalski et al.[2], and about 5 times higher than that of MPN, the synthetic substrate.

The effect of methanol on the hydrolysis of MPN by sialidase is shown in Fig. 7. Increase in methanol concentration does not significantly affect the release of 3-methoxyphenol from the substrate but decreases the amount of free Neu5Ac. This means that other nucleophiles than water can attack the *E-S* complex. This leads to the following kinetic scheme for the solvolysis:



$$E + S \xrightarrow{K_s} ES \xrightarrow{k_2} ES' \begin{cases} \xrightarrow{k_3[H_2O]} E + P_1 + P_2 \\ \xrightarrow{k_4[MeOH]} E + P_1 + P_3 \end{cases}$$

in the equation, E is the free enzyme, S is MPN, ES' is the intermediate in which a Neu5Ac oxocarbonium ion is bound to the enzyme[29],

P_1 is 3-methoxyphenol, P_2 is Neu5Ac and P_3 is Neu5Ac α-methylglycoside. Since methanol has no effect on the release of 3-methoxyphenol (Fig. 6), k_2 has to be smaller than k_3 or k_4. Consequently, $K_{cat}\,p_1 = k_2$, $K_s = K_m$ (app) and the release of 3-methoxyphenol is the rate-determining step for the solvolysis of MPN.

Discussion

Most published methods for the isolation and purification of bacterial sialidases use gel filtration and anion exchange chromatography as essential purification steps[14,15,18,30]. By extending to these conventional separation techniques the FPLC medium pressure chromatography system we were able to purify the *Clostridium perfringens* sialidase from a protein-rich culture medium to homogeneity in a yield of 0.13 mg/*l*. The elution profile showed that there is no substantial ground for the suggestion of Nees et al.[10] that *Clostridium perfringens* sialidase could possibly show heterogeneity characteristic. The purification method presented here provides an homogeneous enzyme preparation. The enzyme had an isoelectric point of about 4.7. The measured K_m values showed the enzyme to have a greater affinity for the synthetic substrates than for the trisaccharides (Table 2), but the rates of hydrolysis for these substrates are lower than for the trisaccharides. The same feature was reported by Corfield et al.[31] with respect to sialyllactose and Neu5Acα(2-6)-GalNAc.

The ability of glycohydrolases to catalyze transglycosylation reactions is well known [4,32]. So far, this property had not been shown for *Clostridium perfringens* sialidase before. The kinetic scheme for the solvolysis shows that the release of 3-methoxyphenol is the rate determining step for the solvolysis of MPN.

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