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Purification and characterization of a sialidase from *Clostridium chauvoei* NC08596

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The sialidase secreted by *Clostridium chauvoei* NC08596 was purified to apparent homogeneity by ion-exchange chromatography, gel filtration, hydrophobic interaction-chromatography, FPLC ion-exchange chromatography, and FPLC gel filtration. The enzyme was enriched about 10 200-fold, reaching a final specific activity of 24.4 U mg^{-1}. It has a relatively high molecular mass of 300 kDa and consists of two subunits each of 150 kDa. The cations Mn^{2+}, Mg^{2+}, and Ca^{2+} and bovine serum albumin have a positive effect on the sialidase activity, while Hg^{2+}, Cu^{2+}, and Zn^{2+}, chelating agents and salt decrease enzyme activity. The substrate specificity, kinetic data, and pH optimum of the enzyme are similar to those of other bacterial sialidases.

Keywords: sialidase (neuraminidase), purification, properties, *Clostridium chauvoei*

Abbreviations: FPLC, fast protein liquid chromatography; NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; MU-Neu5Ac, 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid; buffer A, 0.02 M piperazine, 0.01 M $CaCl_2$, pH 5.5; buffer B, 0.02 M piperazine, 0.01 M $CaCl_2$, 1.0 M NaCl, pH 5.5; buffer C, 0.1 M sodium acetate, 0.01 M $CaCl_2$, pH 5.5; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Neu5Ac, *N*-acetylneuraminic acid; BSM, bovine submandibular gland mucin; GD1a, IV^{3}Neu5Ac, II^{3}Neu5Ac-GgOse$_4$Cer; GM1, II^{3}Neu5Ac-GgOse$_4$Cer; MU-Neu4,5Ac$_2$, 4-methylumbelliferyl-α-D-*N*-acetyl-4-*O*-acetylneuraminic acid; TLC, thin-layer chromatography; HPTLC, high performance thin-layer chromatography; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl-ethen)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; Neu5Ac2en, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; IEF, isoelectric focusing; IEP, isoelectric point.

Sialidases (neuraminidases; EC 3.2.1.18) are widespread among viruses, bacteria, and animals [1, 2]. In bacteria, which often additionally possess acylneuraminate pyruvate-lyase ('aldolase', EC 4.1.3.3.), sialidase permits the use of sialic acids as a source of energy and carbon [1]. As most of the bacteria producing sialidases are pathogenic in man or other mammals, it has been suggested that sialidases play an important role in the pathogenicity of certain infections [3, 4].

Although a large number of animal and bacterial species possess sialidase, only a few sialidases have been studied in detail. Regarding clostridial species, the enzymes of *Clostridium perfringens* [5], *Clostridium sordellii* [6], and *Clostridium septicum* [Rothe Beate, Rothe Bernd, Roggentin P, Schauer R, in press] have already been purified and investigated. The aim of this study was to isolate and characterize the sialidase from *Clostridium chauvoei*, which causes blackleg in cattle and sheep [7]. Furthermore, by this method a sufficient amount of pure enzyme can be obtained for N-terminal sequencing, a prerequisite for getting deeper insight into the gene family of bacterial sialidases.

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Materials and methods

Bacterial strains

C. chauvoei strains NC08070, NC08361, and NC08596 were purchased from NCTC; strains E1, E2, and E8 were obtained from the Untersuchungsinstitut 1 des Sanitätsdienstes der Bundeswehr (D-2300 Kiel) and type strain 10092^T from ATCC.

Cultivation

In order to find the medium for maximum sialidase production, the following culture media were tested without (−) or with the supplement of sialic acids [0.1 mM (+) and 0.3 mM (3+)] bound to sialoglycopeptides prepared from edible bird nest substance [5]: Brain Heart Infusion (Difco, Detroit, MI, USA), Cooked Meat Medium (Difco), Fluid

Thioglycolate Medum (Difco), Heart Infusion Broth (Difco), Nutrient Broth (Serva, Heidelberg, Germany), Todd Hewitt Broth (Difco), and Tryptic Soy Broth (Difco). For sialidase production, cells were grown anaerobically at 37°C in Cooked Meat Medium (−). After multiplication of the bacteria by stepwise inoculation of 10 ml and 1 l, the main culture was incubated for 72 h and the supernatant decanted from the granular sediment of the medium. The sediment was washed with an equal volume of water and decanted once again. These supernatants were combined and centrifuged (10 000 × **g**, 20 min, 4°C) immediately before isolation of the enzyme.

Sialidase purification

Unless stated otherwise, all purification steps were performed at room temperature. Enzyme activity was routinely tested with MU-Neu5Ac, as described below. The combined supernatants (6 l) were adjusted to pH 5.5 and applied to a column (10 × 9 cm) containing Q-Sepharose fast flow (Pharmacia, Freiburg, Germany). The effluent was discarded and the column washed with 2 l of buffer A. The enzyme was eluted with a linear gradient (2 l) of 0–1 M NaCl in buffer A. Fractions containing sialidase activity higher than 50 mU ml^{-1} were pooled and concentrated by pressure dialysis at 4°C on ultrafilters (SM 14669; Sartorius, Göttingen, Germany) with a molecular weight limit of 100 000 in a 350 ml or 50 ml Amicon cell. The concentrated sialidase solution (8 ml) was filtered on a Sephacryl S-300 superfine column (2.4 × 86 cm, Pharmacia), equilibrated and run (7.5 ml h^{-1}) in buffer C at 4°C. Active fractions were collected, concentrated as above and applied to an FPLC Alkyl Superose (HR 5/5, Pharmacia) in aliquots of 2 ml after 1:10 dilution with buffer C saturated to 40% with $(NH_4)_2SO_4$. The column was equilibrated with the same buffer before applying the protein solution and washing the column for 30 min with the equilibration buffer. The sialidase was eluted by a linear, negative $(NH_4)_2SO_4$ gradient (40–0% in 60 min) in buffer C at a flow rate of 0.5 ml min^{-1}. Fractions containing sialidase activity higher than 75 mU ml^{-1} were collected, concentrated as above, dialysed against water in the Amicon cell and concentrated in Centricon 10 tubes (Amicon) to a volume of 200 µl. After 1:4 dilution with buffer A the sample (800 µl) was applied to a FPLC Mono Q column (HR 5/5, Pharmacia). The column was equilibrated with the same buffer and the chromatography was carried out at a flow rate of 0.5 ml min^{-1}. The enzyme was recovered in a stepwise gradient of buffer B in buffer A (10 min 100% A; 2 min 0–15% B; 10 min 15% B; 40 min 15–25% B, 5 min 25–100% B) Fractions with sialidase activity were combined and concentrated to 0.2 ml. After 1:3 dilution with buffer C, 0.6 ml of the sialidase solution was applied to a Superose 6 column (HR 10/30, Pharmacia) equilibrated with buffer C, and the enzyme eluted by using the same buffer at a flow rate of 0.25 ml min^{-1} and collecting fractions of 0.25 ml. Active fractions were pooled, dialysed against water and concentrated as described above.

Protein was routinely measured by absorption at 280 nm (A_{280} of 1 = 1 mg protein ml^{-1}), and in the pools from sialidase purification steps additionally by the method of Lowry *et al.* as modified by Peterson [8].

SDS-PAGE of the sialidase samples was carried out as described by Laemmli [9], using a gradient separation gel (6–12% T, 3.75% C; 16 × 18 × 0.2 cm). Running conditions were 20–30 mA/gel for 6 h at 13°C. Gels were silver-stained by the method of Heukeshoven and Dernick [10]. Proteins for calibration of the molecular weight are listed in Fig. 5.

Sialidase assay

During enzyme isolation, sialidase activity was assayed by the method of Potier *et al.* [11] using 10 µl of sample, 80 µl of buffer C and 10 µl of 1 mM MU-Neu5Ac as substrate. This glycoside was synthesized according to Warner and O'Brien [12] with modifications by Berg *et al.* [13]. The reaction was stopped after 10 min incubation at 37°C by addition of 0.9 ml 0.133 M glycine buffer, pH 10, containing 60 mM NaCl and 40 mM Na_2CO_3. The fluorescence of released 4-methylumbelliferone was determined in a Perkin Elmer filter-spectrofluorimeter (M-1000) using excitation at 365 nm and emission at 450 nm. The instrument was calibrated with 4-methylumbelliferone standard solutions. One unit of enzyme activity was defined as 1 µmol substrate hydrolysed per min.

Substrate specificity and kinetic data

For the investigation of substrate specifity the following natural substrates were tested: Neu5Ac-α(2-3)-lactose, Neu5Ac-α(2-6)-lactose isolated from bovine colostrum [14], colominic acid (Sigma, Deisenhofen, Germany), fetuin (Sigma), BSM [15] as well as the gangliosides GD1a, and GM1 and a ganglioside mixture isolated from bovine brain, as previously described [16]. The reactivity of the sialidase with 4-*O*-acetylated sialic acid was investigated using the synthetic substrate MU-Neu4,5Ac_2, which was kindly provided by H. Ogura [17]. The sialic acid content of these substances was measured by microadaption of the orcinol/Fe^{3+}/HCl-reaction [18]. The assays were conducted in buffer C at 37°C in a total volume of 0.1 ml, containing 1 mM sialic acid bound to sialyllactoses or glycoconjugates and 10 µl of sialidase solution. The activity of the sialidase solution used for the sialyllactoses, colominic acid, fetuin, BSM, and the mixture of bovine brain gangliosides was 17.7 mU ml^{-1} as determined with MU-Neu5Ac. The enzymatic reaction was followed up to 60 min by determination of free sialic acid using the periodic acid/thiobarbituric acid assay [18]. GD1a and GM1 were tested with 79 mU ml^{-1} for 40 min and additionally analysed by TLC as described in [16] after extraction into a 3:1 mixture of chloroform and methanol. MU-Neu4,5Ac_2 was tested in a total volume of 12 µl at a final concentration of 4.2 mM with 13 mU ml^{-1} sialidase. After incubation for 1 h, the reaction products

were analysed by HPTLC on glass-backed kieselgel plates (Merck) using isopropanol: water:glacial acetic acid, 30:8:1 by vol, as mobile phase [19]. The methylumbelliferone fluorescence was measured with a CD60 densitometer (DESAGA, Heidelberg, Germany) with excitation at 364 nm and an emission wavelength longer than 450 nm, after hydrolysis of the MU-glycoside by spraying the plates with 1 M formic acid and heating at 100°C for 10 min.

For determination of K_M and v, kinetic data were fitted to the Michaelis–Menten equation by non-linear regression with the program Enzfitter (Elsevier Biosoft, Oxford, UK).

Modification of sialidase activity

The influence of various substances on the activity of the pure enzyme was tested at the following final concentrations: Ca^{2+}, Mg^{2+}, and Mn^{2+} between 1 and 30 mM, Co^{2+} and Zn^{2+} between 0.5 and 10 mM, EDTA and EGTA at 1 mM, Hg^{2+} between 1 and 5 μM, Cu^{2+} between 0.1 and 10 mM, NaCl between 0.05 and 0.6 M, BSA between 3.0 and 800 μg ml^{-1}, the sialidase inhibitors [20] Neu5Ac2en between 0.01 and 2 mM, *N*-(4-nitrophenyl)oxamic acid between 0.01 and 2 mM, and Neu5Ac between 0.01 and 3.0 mM. The assays (0.1 ml final volume) containing 10 μl sialidase solution (30.0 mU ml^{-1}), and 10 μl 1 mM MU-Neu5Ac solution, were carried out in buffer C. The influence of divalent cations was tested in 0.1 M sodium acetate buffer, pH 5.5. Enzyme activity was measured after 15 min of preincubation without substrate at 37°C with the fluorescence assay described.

Other properties

Sialidase activities were measured at temperatures between 4 and 60°C in buffer C, containing 10 μl purified sialidase (22 mU ml^{-1}) and MU-Neu5Ac after 15 min preincubation at the desired temperature. The activity was also measured at 37°C at various pH values between pH 3.0 and 7.5 using steps of 0.5 pH units in 0.1 M sodium acetate/acetic acid from pH 3.0 to 7.5. The isoelectric point was determined by preparative isoelectric focusing as described by Roggentin *et al.* [6], with the modification that the gel was prefocussed for 1 h and the enzyme sample was not freeze-dried but concentrated by ultrafiltration and dialysed against water before applying at different points diagonally to the granulated carrier (IEF-Sephadex, Pharmacia). The gel was run for only 1.5 h. For analysis, the gel was sprayed with a 0.1 M MU-Neu5Ac solution according to the method of Berg *et al.* [13] and the pH range to which the enzyme migrated was measured according to [6]

Results and discussion

Isolation procedure

In order to determine the optimal conditions for obtaining large amounts of enzyme protein, different strains, culture media and incubation times were investigated. In comparison to Cooked Meat Medium the enzyme production in all the other media was very low. The addition of sialoglycopeptides did not increase sialidase production. After 96 h incubation time, the amounts of sialidase produced by E2, E8, and NC08596 in Cooked Meat Medium were approximately the same (Fig. 1). Strain NC08596 was chosen for the purification of the enzyme, because it is obtainable from an international collection.

After sedimentation of the cells, the total enzyme activity was found in the supernatant. Using the purification procedure described, the enzyme could be enriched over 10000-fold and 5 units of pure enzyme were obtained from one 6 l culture. The results of the purification procedure are summarized in Table 1. The values represent the means obtained from the processing of four 6 l cultures. As already described [6, 21], the use of a "fast flow" ion-exchange resin for the first purification step resulted in the removal of contaminating protein under mild conditions and a 10-fold purification. While about 125% of the activity of the culture supernatant was found in the pool after ion-exchange chromatography, up to 188% was obtained after gel filtration, which was conducted as a second purification step. This apparent increase in activity, which was oserved also during the isolation of other sialidases [5, 6, 22], may be due to the gradual removal of substrates, e.g. sialoglycoconjugates present in the culture medium and interfering with the MU-Neu5Ac assay. The activity values obtained for the culture supernatant and the pool after ion-exchange chromatography therefore do not represent the true total activity of sialidase present.

Using FPLC hydrophobic-interaction chromatography, the enzyme was further enriched 19-fold, but this step also led to a marked decrease of enzyme yield (Fig. 2). Further fractionation of the concentrated and dialysed pool of sialidase by FPLC ion-exchange chromatography (Fig. 3) resulted in an enzyme preparation containing only two additional contaminating protein bands on SDS-PAGE. These were finally removed by FPLC gel filtration (Fig. 4) giving a sialidase preparation, which exhibited a single band on SDS-PAGE (Fig. 5).

Ammonium sulfate precipitation, preparative isoelectric focusing, FPLC metal-chelating chromatography, and FPLC chromatofocusing were tested as additional methods, but proved to be unsuitable for purifying this sialidase.

Sialidase properties

The sialidase isolated from *C. chauvoei* is rather stable. During five cycles of freezing and thawing no loss of activity was measured. The activity remained unchanged at 37°C in buffer C for at least 1 h. The molecular weights estimated by SDS-PAGE and FPLC gel filtration were 150000 and 300000, respectively. It is therefore concluded that the protein consists of two subunits of identical mass. This is the first bacterial sialidase reported to be composed of

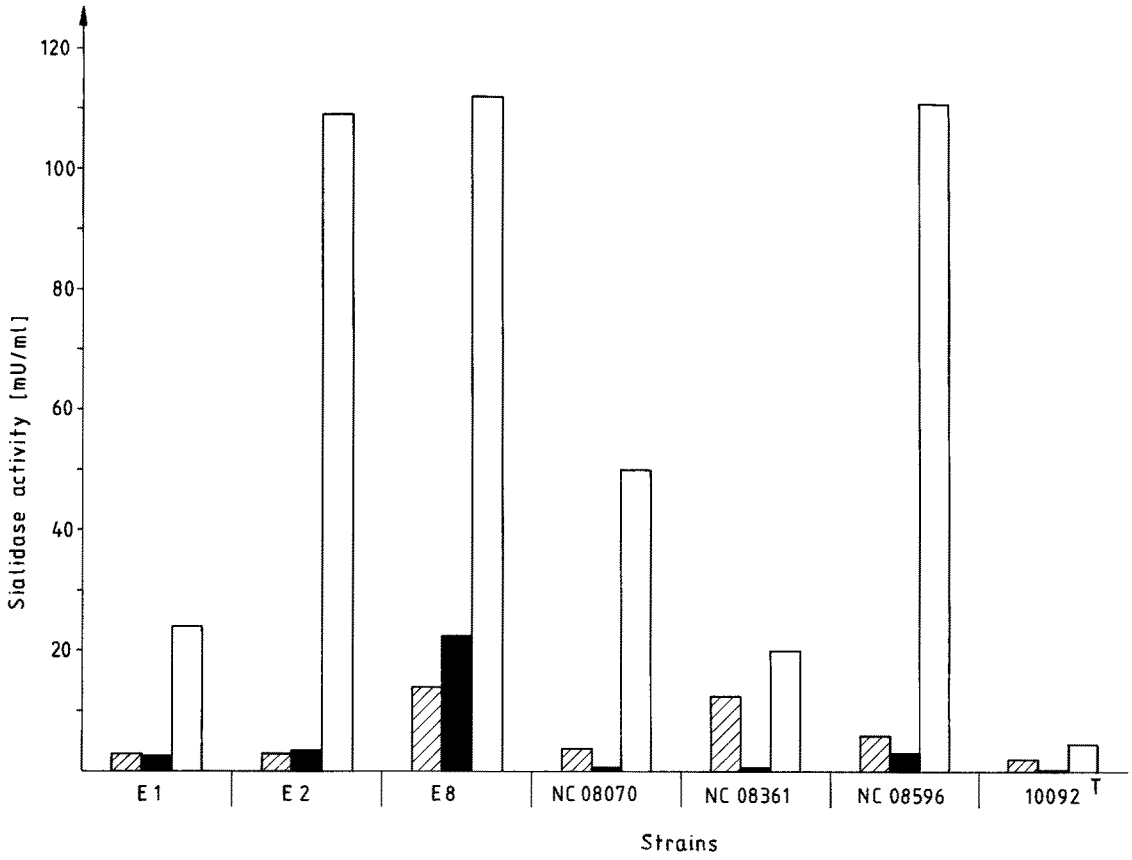

Figure 1. Soluble sialidase activity formed by seven strains of *C. chauvoei* in three different culture media after 96 h incubation at 37°C with (+, 3+) or without (−) the addition of sialoglycopeptides. For details see the Materials and methods section. Hatched columns: Heart Infusion Broth +; black columns: Heart Infusion Broth 3+; empty columns: Cooked Meat Medium −.

Table 1. Purification of sialidase from *C. chauvoei* NC08596. The values are means from the processing of four 6 l cultures.

Purification step	*Specific activity* [U mg^{-1}]	*Purification* [*factor*]	*Recovery* [%]
Supernatant	0.0024	1	100
1. Ion-exchange chromatography	0.024	10	125
2. Gel filtration	0.094	39	188
3. Hydrophobic interaction chromatography (FPLC)	1.8	750	28.4
4. Ion-exchange chromatography (FPLC)	10.4	4 333	21.8
5. Gel filtration (FPLC)	24.4	10 167	5.3

subunits. Complex structures are only known for viral sialidases [23] and for one animal (starfish *Asterias rubens*) enzyme [24] up to now. Other bacterial sialidases with relatively high molecular weight have been found in *Streptococcus* K6646 [25], *Actinomyces viscosus* [21] and *C. septicum* [Rothe Beate, Rothe Bernd, Roggentin P, Schauer R, in press].

The sialidase is active over a broad range of temperatures (4–50°C) and pH values (4–7.5) with optima at 37°C and pH 5.5, respectively. The isoelectric point is in the range of pH 3.4. The possibility must be considered that the sialidase has an IEP even lower than pH 3.4, because its activity is very sensitive to low pH values and by the MU-Neu5Ac spray test only active enzyme is detectable. The relatively low IEP is a hint that the enzyme consists of a high proportion of negatively charged amino acids.

In view of the reported influence of certain metal ions on sialidases from different sources [23], the effect of various cations, EDTA and EGTA on the *C. chauvoei* enzyme was investigated. As shown in Table 2, the sialidase activity was

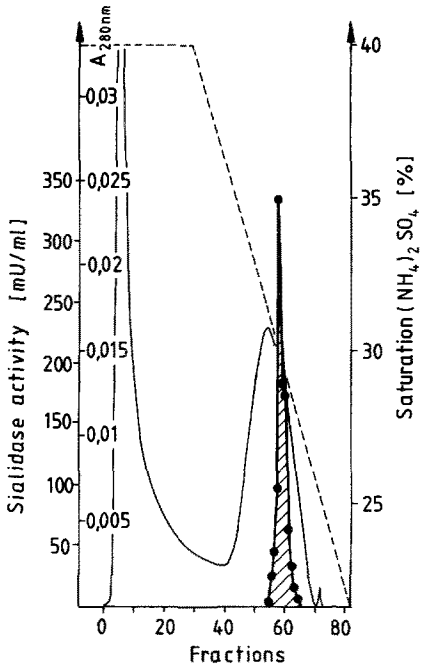

Figure 2. Hydrophobic interaction chromatography (FPLC) of the sialidase pool from gel filtration. Proteins retained (——) were eluted by a linear, negative $(NH_4)_2SO_4$ gradient (– – –). The hatched area represents fractions containing sialidase activity.

slightly stimulated by Ca^{2+}, Co^{2+}, Mg^{2+}, and Mn^{2+}, ions which are known to activate the enzymes of *Vibrio cholerae* [23], *Streptococcus* K6646 [25], and *Bacteroides fragilis* [26]. Accordingly, the activity decreased by the addition of EDTA or EGTA. Cu^{2+}, Zn^{2+}, and Hg^{2+} diminished the sialidase activity to 50% at concentrations of 0.75 mM, 0.5 mM, and 0.001 25 mM, respectively. Inhibition of enzyme activity by mercury ions was found with all sialidases tested [2] and indicates the presence of essential SH groups. Moreover, the enzyme was inhibited by Neu5Ac, *N*-(4-nitrophenyl)oxamic acid, and Neu5Ac2en in an increasing extent, 50% values being achieved by 2.4, 0.41, and 0.01 mM concentrations, respectively, This behaviour is exhibited by a variety of sialidases from other sources, too [1, 2].

Enzyme activity was slightly increased by the addition of protein (BSA), 100 μg ml^{-1} leading to maximum activity (134%). On the other hand, enhanced ionic strength decreased sialidase activity, 600 mM NaCl, for example, by 32%. Both of these effects are in accordance with the behaviour of *C. sordellii* sialidase [6].

The relative hydrolysis rates of the natural substrates tested are summarized in Table 3. The enzyme shows highest activity towards saponified BSM, not containing *O*-acetyl groups, and fetuin, respectively. Therefore, according to a classification of von Nicolai [27] and Corfield *et al.* [2], *C. chauvoei* sialidase belongs to the second main group of sialidases which includes enzymes prefering glycoproteins as substrates. The hydrolysis rate of sialyllactoses is considerably lower, however, a preference for the α(2-3)- over the α(2-6)- and α(2-8)-linkages was observed. With the exception of the enzymes from *Arthrobacter ureafaciens* [28] and *Streptococcus pneumoniae* [2], all sialidases of bacterial and of other origin prefer α(2-3) bonds when compared with α(2-6) linkages [1, 2, 23]. Remarkably, colominic acid,

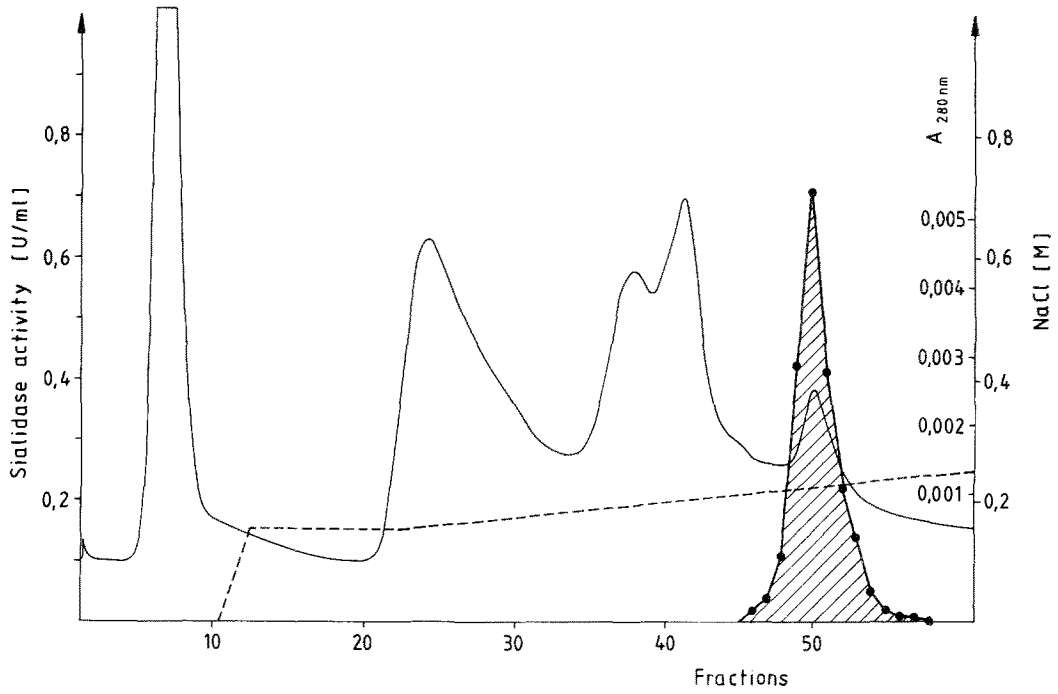

Figure 3. FPLC on ion-exchange resin (Mono Q) of the active pool from hydrophobic interaction chromatography. Elution was carried out by a stepwise gradient of NaCl (– – –). Protein was measured at 280 nm (——). Sialidase activity was found in the hatched area.

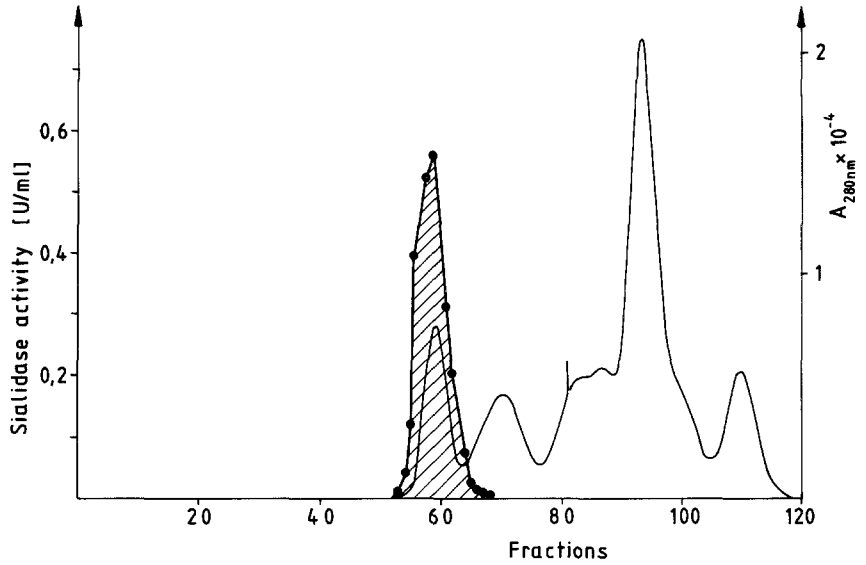

Figure 4. FPLC gel filtration (Superose 6) of the active pool after ion-exchange FPLC. Sialidase activity was found in the hatched area. Protein content is also indicated (——).

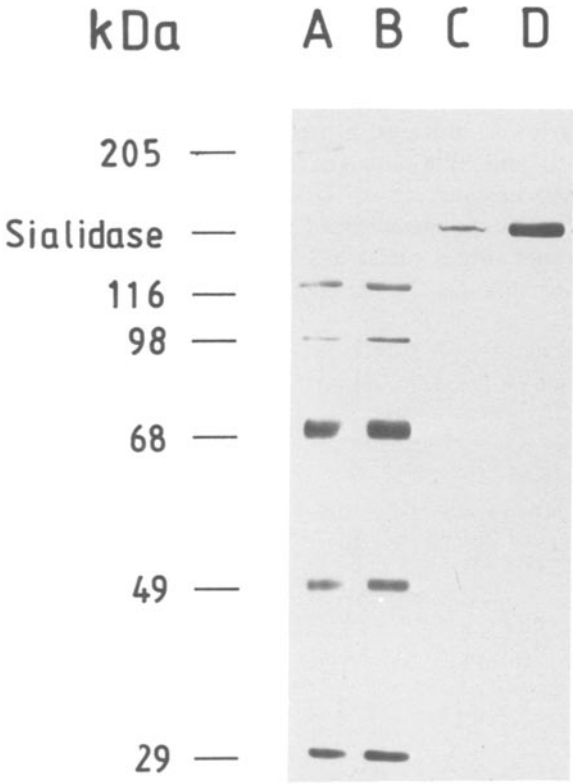

Figure 5. SDS-PAGE of *C. chauvoei* sialidase finally purified by FPLC gel-filtration. Lane A and B: molecular mass markers (SDS-MW Kit 200, Sigma); lane C: sialidase (0.22 µg protein); lane D: sialidase (0.48 µg protein).

having α(2-8) linkages, is hydrolysed at a very low rate. The susceptibility of the ganglioside mixture is relative high when compared to other bacterial sialidases, with the exception of the enzymes from *Actinomyces viscosus* [21] and *Arthrobacter ureafaciens* [2]. The conversion of GD1a

Table 2. Effect of some divalent cations and chelators on the activity of sialidase isolated from *C. chauvoei* NC08596. For details see the Materials and methods section.

Test substance	*Concentration of max. effect* [mM]	*Sialidase activity* [% *of control*]
$CaCl_2$	20	129.6
$CoCl_2$	1.5	115
$MgCl_2$	20	120
$MnCl_2$	3	129
EDTA[a]	1.0	15
EGTA[a]	1.0	28

[a] Only this concentration was tested.

Table 3 Relative hydrolysis rates of natural substances, incubated at 1 mM sialic acid concentration with *C. chauvoei* NC08596 sialidase for 20 min at 37°C. For details see text.

Substance	*Relative hydrolysis rate* [%]
BSM (saponified)	100
Fetuin	90.3
Neu5Ac-α(2-3)-lactose	51.0
Neu5Ac-α(2-6)-lactose	31.6
BSM (native)	31.2
Ganglioside mixture from bovine brain	13.6
Colominic acid	3.8

Table 4. Kinetic properties of the sialidase from *C. chauvoei* NC08596. For details see text.

Substance	*Kinetic properties*	
	K_M [mM]	v[U mg^{-1}]
Neu5Ac-α(2-3)-lactose	2.2	0.9
Neu5Ac-α(2-6)-lactose	2.0	0.3
MU-Neu5Ac	0.09	35

to GM1 was complete, as shown by TLC, after 40 min incubation with a relatively high amount of the *C. chauvoei* sialidase (79 mM ml^{-1}). GM1 was investigated under the same conditions, but was not attacked at all. The behaviour towards 4-*O*-acetylated sialic acid was investigated with MU-Neu4,5Ac$_2$ by HPTLC. In accordance with other bacterial and mammalian sialidases [1, 2, 19, 23], 4-*O*-acetylated sialic acid was not released by the *C. chauvoei* enzyme. Michaelis–Menten kinetics were determined for three substrates as shown in Table 4, indicating that the synthetic substrate MU-Neu5Ac has a higher affinity to the enzyme and is hydrolysed with a higher rate than the natural substrates tested. The K_M values for α(2-3)- and α(2-6)-sialyllactoses are in the range described for other bacterial sialidases [2]. Although the K_M values for both oligosaccharides are similar, the preference of substrates with α(2-3)-linked sialic acid is pointed out by a higher V-value for Neu5Ac-α(2-3)-lactose.

Altogether the characteristics of *Clostridium chauvoei* sialidase such as substrate specifity, stability over a broad temperature and pH range, and the possibility to stimulate enzyme production by the Cooked Meat Medium used, comply with the requirements described for sialidases as pathogenic factors [3, 29]. This gives support to the hypothesis [3] that the *C. chauvoei* sialidase plays a role in the spreading of blackleg in the infected host. With regard to the evolution of sialidases [1], *C. chauvoei* sialidase may be of interest because of its unusually high molecular weight and subunit structure. Further investigations will show whether conserved sequences already known for some bacterial and viral sialidases [30] are detectable also in the *C. chauvoei* sialidase gene.

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