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Isolation and properties of the natural and the recombinant sialidase from *Clostridium septicum* NC 0054714

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The natural sialidase of *Clostridium septicum* was purified and characterized in parallel with the recombinant enzyme expressed by *Escherichia coli*. The two enzymes exhibit almost identical properties. The maximum hydrolytic activity was measured at 37 °C in 60 mM sodium acetate buffer, pH 5.3. Glycoproteins like fetuin and saponified bovine submandibular gland mucin, most of them having α(2-6) linked sialic acids, are preferred substrates, while sialic acids from gangliosides, sialyllactoses, or the α(2-8) linked sialic acid polymer (colominic acid) are hydrolysed at lower rates. α(2-3) Linkages are more rapidly hydrolysed than α(2-6) bonds of sialyllactoses. The cleavage rate is markedly reduced by O-acetylation of the sialic acid moiety. These properties are similar to those of other secreted clostridial sialidases. The enzyme exists in mono-, di- and trimeric forms, the monomer exhibiting a molecular mass of 125 kDa, which is close to the protein mass of 111 kDa deduced from the nucleotide sequence of the cloned gene.

Keywords: sialidase (neuraminidase), cloned and natural enzyme, isolation, comparison of properties, *Clostridium septicum*

Abbreviations: BSM, bovine submandibular gland mucine; CMM, cooked meat medium; EDTA, ethylenediaminetetraacetic acid; FPLC, fast performance liquid chromatography; LB, Luria-Bertani; MU-Neu5Ac, 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; Neu4,5Ac$_2$, *N*-acetyl-4-*O*-acetylneuraminic acid; pI, isoelectric point; SDS, sodium dodecyl sulfate.

Introduction

Sialidases (neuraminidases; EC 3.2.1.18) of diverse forms are produced by some viruses, bacteria, fungi, protozoa, and the animals of the deuterostomate lineage [1, 2]. Most microorganisms and viruses have close contact to these animals, thereby using sialidase for nutrition [3], adhesion [4] or as a spreading factor during infection [5]. Sialidase catalyses the hydrolysis of α-glycosidically bound sialic acids which frequently are terminal constituents of animal oligosaccharides, glycoproteins and glycolipids [6]. The removal of sialic acids alters the structure and function of the sialoglycoconjugates, thus enabling pathogen bacteria to elicit further destruction of tissue by secreted proteases, lipases and other glycosidases. It is therefore suggested that sialidases play an important role in pathogenesis [7–9]. *Clostridium septicum* like *Clostridium perfringens* and other clostridial species, causes 'haemorrhagic-gelatinous gas oedema' in man. The infection is originated by spores which germinate in contaminated wounds with insufficient blood supply, from which the bacteria rapidly spread into the tissues and invade the bloodstream [10].

Although a large number of bacterial species possess sialidases, only a few of them have been studied in detail. Regarding clostridial species, the sialidases of *Clostridium perfringens* [11], *C. sordellii* [12] and *C. chauvoei* [13] have already been purified and investigated. The two bacterial species, *C. chauvoei* and *C. septicum*, are very similar phenotypically. They differ only in the fermentation of salicine and the hydrolysis of cellobiose, trehalose and sucrose [14].

The aim of this study was to isolate and characterize the natural sialidase of *C. septicum* as well as the recombinant gene product expressed in *E. coli* [15]. A comparison of the two enzymes may prove the identity of the cloned sialidase. Furthermore, the resulting properties are compared with those of other sialidases, especially with the enzyme of the closely related *C. chauvoei*, to learn about the molecular relationship of bacterial sialidases.

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Materials and methods

Bacterial strains and cultivation

The *C. septicum* strain NC 0054714 was purchased from the National Collection of Type Cultures, London. It was cultivated in Cooked Meat Medium (CMM, Difco, Detroit, MI, USA), which induces a maximum of sialidase production in comparison with media tested earlier [15]. Cells were multiplied by stepwise inoculation of 10, 40, 450 and 5 × 900 ml CMM, and were grown anaerobically at 37 °C for 24 h during the first multiplication steps and 72 h in the last step. The final culture was filtered through paper filters to remove the meat particles, which were washed with distilled water at a volume corresponding to the liquid phase of the medium. The filtered fractions were centrifuged (10 000 × **g**, 4 °C, 20 min) to remove the cells. The combined supernatants (6 l) were used for sialidase isolation.

The *E. coli* clone BJ7 bearing the sialidase gene of *C. septicum* was constructed as described earlier [15]. For sialidase production, the *E. coli* cells were cultivated in LB-medium [16] supplemented with 100 mg l^{-1} ampicillin. After 1:300 inoculation, the main cultures (5 × 1 l) were grown at 37 °C for 18 h under vigorous shaking. The cells were sedimented by centrifugation (10 000 × **g**, 4 °C, 10 min) and resuspended in 500 ml lysozyme buffer [1 mg ml^{-1} lysozyme (Boehringer-Mannheim, Germany) in 1% (w/v) glucose, 0.2% EDTA and 0.3% Tris, pH 8.0] [16]. Sialidase was liberated by stirring the cell suspension for 1 h at 37 °C. The cells were sedimented by centrifugation (10 000 × **g**, 4 °C, 20 min) and the supernatant (480 ml) was used for sialidase isolation.

Purification of the sialidases

Unless stated otherwise, all purification steps were carried out for both sialidases.

Protein content of enzyme solutions was routinely measured by absorption at 280 nm (A_{280} of 1 = 1 mg protein per ml). In enzyme solutions used for further experiments, protein was additionally determined by a colorimetric assay [17].

As a first step, each supernatant was adjusted to pH 6.0, which was the optimum pH for binding the sialidases to an anion exchange resin. The lysozyme supernatant of *E. coli* clone BJ7 and the culture supernatants of *C. septicum*, containing about 150 U sialidase activity each, were applied to a Q-Sepharose Fast Flow column (10 × 11 cm, Pharmacia, Freiburg, Germany) at 20 °C. The effluent was discarded and the column washed with 2 l 20 mM piperazine buffer, pH 6.0. Sialidase was eluted by a linear gradient of 0–1 M KCl in 2 l of the same buffer. Fractions containing sialidase activity higher than 50 mU ml^{-1} were pooled, and concentrated using ultrafilters (20 kDa molecular mass limit, Sartorius, Göttingen, Germany) in a 350 ml dialysis cell (Amicon, Witten, Germany) at 4 °C. The resulting sialidase solution (11 ml) was filtered on a Sephadex G-200 column (5 cm × 140 cm), equilibrated in 60 mM sodium acetate buffer, pH 5.3, at 4 °C (10 ml h^{-1}). This step was used exclusively for the natural *C. septicum* enzyme. Active fractions were pooled and concentrated as described above.

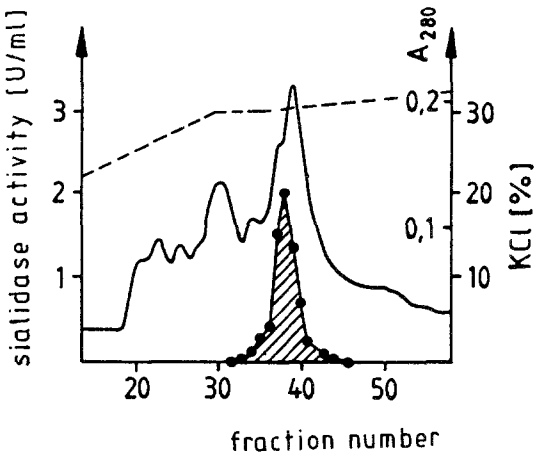

Figure 1. Elution profile of the recombinant sialidase of *C. septicum* after FPLC ion exchange rechromatography: sialidase activity (mU ml^{-1}), •, hatched area; protein concentration (mg ml^{-1}), ——, open area; salt gradient (%), – – –.

The concentrated solutions of natural and recombinant enzyme were diluted 1:2 with 20 mM piperazine buffer, pH 6.0, which was also used for equilibration of the FPLC ion exchange column (Mono Q, HR 10/10, Pharmacia). After application at 20 °C, the sialidase was eluted by a nonlinear KCl gradient at a flow rate of 0.5 ml min^{-1} as demonstrated in Fig. 1. Fractions containing sialidase activity higher than 600 mU ml^{-1} were combined, diluted 1:2 with buffer, and rechromatographed under identical conditions. Eluted fractions containing sialidase activity higher than 750 mU ml^{-1} were collected, concentrated and applied to a Superose-12 gel filtration column (HR 10/30, Pharmacia). The column was equilibrated and run with 20 mM piperazine buffer, pH 6.0, supplemented with 100 mM KCl. The proteins were filtered at a flow rate of 300 µl min^{-1}; 150 µl fractions were collected and stored at −20 °C.

Determination of sialidase properties

Molecular weight. The native sialidases were filtered on a FPLC column (Superose-12, HR 10/30, Pharmacia). Apoferritin (450 000 Da), alcohol dehydrogenase (150 000 Da), bovine serum albumin (66 000 Da) and egg albumin (45 000 Da) were used as molecular weight standards (1 mg ml^{-1}; Pharmacia). Enzyme solutions and molecular weight standards were applied to the column in 100 µl aliquots.

The molecular mass under denaturing conditions was determined by SDS-PAGE [18]. Various conditions for enzyme denaturation, including mercaptoethanol, SDS-contents of 8.4, 15 and 20%, and incubation times at 96 °C of 10, 20 and 30 min were applied. The separation gel (16 × 18 × 0.2 cm) contained 6% acrylamide. Running

conditions were 30–60 mA for 6 h at 14 °C. The separated proteins were silver-stained [19]. The sialidase mass was estimated according to 'large' protein standards (Sigma, Deisenhofen, Germany) run in parallel.

Enzyme stability. The stability of the pure enzymes to freezing and thawing or storage at 4 °C, 20 °C and 37 °C for 24 and 48 h was tested with sialidase solutions (210 mU ml^{-1}) in 60 mM sodium acetate buffer, pH 5.3.

Enzyme stability during storage at various pH values was determined by incubation of sialidase solutions (35 mU ml^{-1}) at 37 °C in 0.1 M sodium acetate buffer (pH 3.06–7.0) and in 0.1 M glycylglycine/glycine buffer (pH 7.5–10.0) at intervals of 0.5 pH units. Sialidase activities were measured every hour.

Sialidase assays and substrate specificity. During enzyme isolation, sialidase activity was assayed [20] with the fluorogenic substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac; Pallmann, Munich, Germany). The test mixture contained 10 µl 1 mM aqueous MU-Neu5Ac solution, 10 µl sialidase sample and 80 µl 60 mM sodium acetate buffer, pH 5.3. The reaction was stopped after 10 min incubation at 37 °C by the addition of 900 µl 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 filter spectrofluorimeter (M-1000; Perkin-Elmer, Überlingen, Germany) 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 at 37 °C.

The hydrolysis of 4-*O*-acetylated Neu5Ac was tested with the synthetic substance MU-Neu4,5Ac_2, a gift from H. Ogura (Tokyo). The substrate specificity was examined with the following natural substrates: Neu5Ac-α(2-3)-lactose, Neu5Ac-α(2-6)-lactose, both isolated from bovine colostrum [21], colominic acid (Sigma), fetuin (Sigma), native [22] or saponified [23] BSM and a ganglioside mixture isolated from bovine brain [24]. The assays were conducted at 37 °C in 60 mM sodium acetate buffer, pH 5.3, in a final volume of 100 µl, containing 1 mM sialic acid bound to the respective glycoconjugate, and 10 µl of a sialidase solution exhibiting an activity of 5 mU ml^{-1}. The enzymatic reaction was followed up to 60 min by determination of free sialic acid with the periodic acid/thiobarbituric acid assay [25].

Kinetic properties. V_{max} and K_M values were determined for the following substrates: fetuin, Neu5Ac-α(2-3)- and Neu5Ac-α(2-6)-lactose in concentrations of 0.1, 0.5, 1.0, 1.5 and 2.0 mM, and for 0.01, 0.05, 0.1, 0.5 and 1.0 mM MU-Neu5Ac. After a 15 min incubation (within the linear reaction) with 25 mU or 5 mU (MU-Neu5Ac) sialidase in 100 µl at 37 °C, the amounts of released sialic acids or MU were measured.

pH and buffer optimum. These were determined with the standard assay in 0.01, 0.1 or 1 M sodium acetate, sodium citrate and sodium phosphate buffer, respectively, ranging from pH 3.0 to pH 7.5 in intervals of 0.5 pH units with enzyme solutions containing 17 mU ml^{-1} sialidase activity. From pH 4.5 to pH 5.5, the activity was measured in intervals of 0.1 pH units in 10–100 mM sodium acetate buffer with enzyme solutions containing 23 mU ml^{-1} sialidase activity.

Temperature optimum. The optimum temperature for hydrolysis was estimated in 60 mM sodium acetate buffer, pH 5.3. Sialidase activity was measured with the standard assay at 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 33 °C, 35 °C, 37 °C, 39 °C, 40 °C, 45 °C and 50 °C after a 15 min preincubation of sialidase solutions (25 mU ml^{-1}).

Alteration of sialidase activity. The influence of the following substances on the activity of the pure enzymes was tested under standard assay conditions (MU-Neu5Ac as substrate) at the final concentrations given in parentheses: (a) common sialidase inhibitors (sialidase activity 22 mU ml^{-1}) Neu5Ac (0.01 and 1.0 mM), Neu5Ac2en (0.01 and 2.0 mM), Hg^{2+} (0.01 and 10 mM), Cu^{2+} (0.1 and 10 mM); (b) ionic strength (sialidase activity: 13 mU ml^{-1}): NaCl and KCl (0.2–1.0 M); (c) divalent cations and a chelator (sialidase activity 15 mU ml^{-1}) Ca^{2+}, Mg^{2+} and Mn^{2+} (1 and 30 mM), Co^{2+}, Ni^{2+} and Zn^{2+} (0.5 and 10 mM), EDTA (0.2 and 10 mM).

Results and discussion

Sialidase purification

Maximum amounts of sialidase were produced by *C. septicum* after 72 h of incubation in CMM. After sedimentation of the cells, the entire enzyme activity was found in the supernatant. The natural enzyme was enriched more than 33 000-fold, resulting in 8.1 units of pure enzyme from 5 l culture (Table 1).

Highest sialidase activity was obtained from the recombinant *E. coli* cells after 18 h of cultivation. From the lysozyme supernatant, the enzyme was enriched over 260-fold, obtaining 19 units of pure enzyme from the cells of 5 l culture (Table 2). The values of the purification steps summarized in Tables 1 and 2 represent the means obtained from the processing of three 5 l cultures of each bacterium.

As described earlier [12, 13, 26, 27], the use of 'fast-flow' ion exchange resins as a first purification step resulted in the removal of large amounts of contaminating proteins, and in a four-fold and 15-fold enrichment of the recombinant and natural enzyme, respectively. Pressure dialysis also proved to be a useful purification method, although this step led to a decrease of enzyme yield (Tables 1 and 2). Gel filtration was shown to be a successful purification method only for the natural enzyme, whereby a large amount of

Table 1. Purification steps for the natural sialidase of *C. septicum*.

Purification step	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U mg^{-1})*	*Purification factor*	*Recovery (%)*
Culture supernatant	605 000	150	0.000 25	1	100
Ion-exchange chromatography	29 000	106	0.003 7	15	71
Pressure dialysis	446	52	0.12	480	35
Gel filtration	21	27	1.3	5200	18
Pressure dialysis	11	21	2.0	8008	14
FPLC ion-exchange chromatography	4	17	4.6	18 420	11
FPLC ion-exchange re-chromatography	2	16	7.0	28 000	11
FPLC gel filtration	1	8.1	8.5	33 600	5

Table 2. Purification steps for the recombinant sialidase of *C. septicum* expressed in *E. coli*.

Purification step	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U mg^{-1})*	*Purification factor*	*Recovery (%)*
Lysozyme supernatant	2496	150	0.06	1	100
Ion-exchange chromatography	484	115	0.24	4	77
Pressure dialysis	168	87	0.52	9	58
FPLC ion-exchange chromatography	14	64	4.5	76	43
FPLC ion-exchange re-chromatography	5	39	7.9	133	26
FPLC gel filtration	1	19	15.8	267	13

contaminating proteins could be separated again. The recombinant enzyme, however, eluted as part of a large peak of contaminating proteins (data not shown).

FPLC ion-exchange chromatography led to further enrichment of both enzymes and resulted in similar specific activities: 4.5 U mg^{-1} for the recombinant sialidase and 4.6 U mg^{-1} for the natural enzyme. Using a suitable salt gradient, large amounts of contaminating proteins could be removed. As already described for the sialidase of *C. sordellii* [12], re-chromatography under identical conditions improved purification and led to specific activities of 7.9 U mg^{-1} for the recombinant enzyme after an overall 133-fold enrichment, and of 7.0 U mg^{-1} for the natural sialidase enriched 28 000-fold. The elution patterns are shown in Figs 1 and 2. These sialidase pools were used to investigate the properties of both enzymes, as the elution profiles exhibited only one peak of activity for each enzyme. Aliquots of these pools were applied to FPLC-gel filtration followed by SDS-PAGE, to allow further purification and to determine the molecular weight of the sialidases. Both enzymes were further enriched by FPLC gel filtration to 15.8 U mg^{-1} for the recombinant sialidase, and 8.5 U mg^{-1} for the natural enzyme, representing the highest specific activities ever obtained. More than 50% of contaminating proteins could be removed in both cases.

Ammonium sulfate precipitation and hydrophobic interaction chromatography were tested as additional methods, but proved to be unsuitable for purification of these sialidases. Preparative isoelectric focusing, which has been described as a useful purification method for other sialidases [12, 27], could not be applied to the sialidase of *C. septicum*, as it exhibits an isoelectric point below pH 3.5 (Beate Rothe,

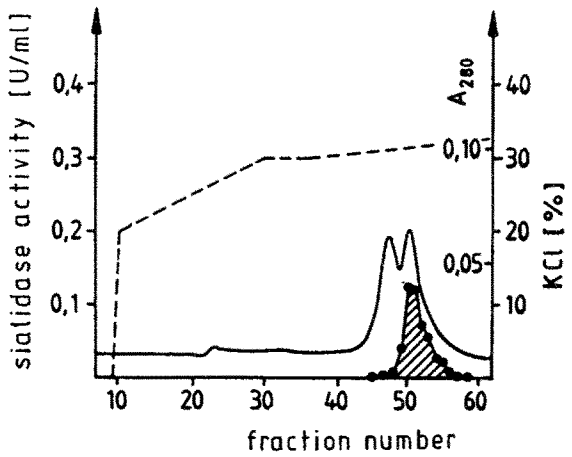

Figure 2. Elution profile of the natural sialidase of *C. septicum* after FPLC ion-exchange re-chromatography. Sialidase activity (mU ml^{-1}), •, hatched area; protein concentration (mg ml^{-1}), ——, open area; salt gradient (%), – – –.

personal communication). A comparable pI at a denaturing pH is described for the sialidase of *C. chauvoei* [13].

Sialidase properties

Unless stated otherwise, the details mentioned resemble the properties of the natural as well as the recombinant sialidase of *C. septicum*.

The determination of the molecular weight by FPLC-gel filtration resulted in three peaks of sialidase activity (Figs 3 and 4) which represent proteins of 380, 210 and 130 kDa (average ±10 kDa). This heterogeneity in size has been recognized earlier (Beate Rothe, personal communication). SDS-PAGE of the separated sialidase peaks after FPLC gel filtration shows identical patterns for both enzyme preparations, revealing proteins with molecular masses of 370, 235 and 125 kDa, respectively (Fig. 5). These results were obtained with every denaturation procedure applied. Why

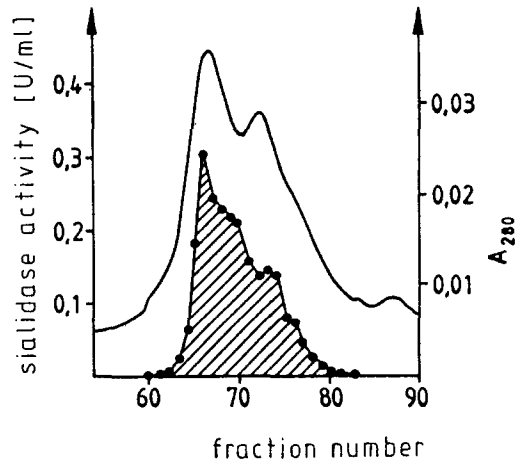

Figure 3. Elution profile of the recombinant sialidase of *C. septicum* after FPLC gel filtration. Sialidase activity (mU ml^{-1}), •, hatched area; protein concentration (mg ml^{-1}), ——, open area.

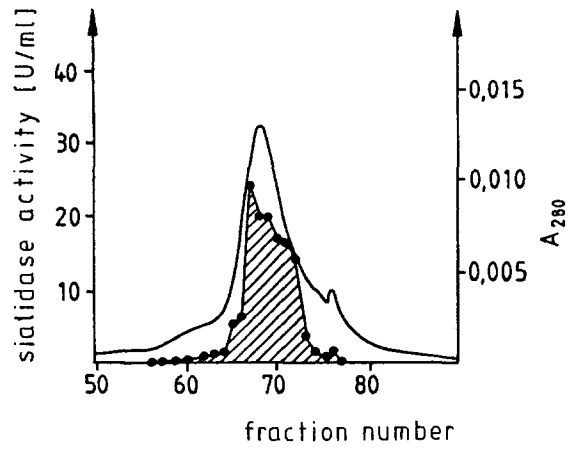

Figure 4. Elution profile of the natural sialidase of *C. septicum* after FPLC gel filtration. Sialidase activity (mU ml^{-1}), •, hatched area; protein concentration (mg ml^{-1}), ——, open area.

the various procedures did not exclusively lead to the monomeric form remains unexplained.

It can be concluded from these data that the recombinant and the natural sialidases of *C. septicum* consist of identical subunits. By using two different bacteria, grown in different media, the presence of identical contaminants is unlikely. Therefore, the identical protein bands visible in silver-stained gels can be caused only by the isolated sialidase. The monomeric form (M_r: 125 kDa, similar to the calculated M_r of 111 kDa, based on the primary structure of the cloned gene) is smaller than the subunit of *C. chauvoei* (M_r: 150 kDa), but larger than the monomeric form of any other clostridial sialidase studied [13]. To our knowledge no other sialidase has been found to exist as a trimer. Up to now, complex tetrameric structures are known for viral sialidases [28] and for the sialidase of the starfish *Asterias rubens* [29]. A composition of two identical subunits has been described for the sialidase of *C. chauvoei* [13], a close relative to *C. septicum* [14]. Descriptions of other animal sialidases reveal that complex structures might consist either of identical or different subunits [9].

The purified enzyme is stable to freezing and thawing like most other clostridial sialidases [11–13]. Between pH 6.0 and pH 6.5, no loss of activity was measured after 6 h at 37 °C. At pH 5.3, the enzyme was stable for 48 h at 4 °C; activity was reduced to 97% at 20 °C and to 20% at 37 °C within 48 h. The addition of 1 M NaCl or KCl to the reaction buffer (60 mM sodium acetate, pH 5.3) reduced the sialidase activity to 64 and 61%, respectively. A comparable effect was also described for the *C. sordellii* sialidase [12].

Using sodium acetate as enzyme buffer, optimum activity was measured at 60 mM concentrations. The influence of various cations, EDTA and Triton X-100 on the *C. septicum* sialidase activity is shown in Table 3. The sialidase activity was slightly stimulated by most of the divalent cations used, but no specific effect was detectable. Accordingly, enzyme activity is decreased by the addition of EDTA. Inhibition of sialidase activity by Hg^{2+} was found with all sialidases investigated so far, and indicates the presence of essential SH groups. Remarkably, the concentration of Cu^{2+} or Hg^{2+}, which was necessary to decrease enzyme activity to 50% (5 mM Cu^{2+} and 0.05 mM Hg^{2+}, respectively) was 100-fold higher than reported for all other sialidases, including the enzyme of *C. chauvoei* [13]. Furthermore, the enzyme was inhibited by *N*-acetylneuraminic acid and Neu5Ac2en, respectively, at an increasing extent. The activity of the recombinant sialidase was decreased by 50% by 1.0 mM *N*-acetylneuraminic acid and the natural one by 0.9 mM *N*-acetylneuraminic acid. 0.1 mM Neu4Ac2en inhibited 96% of the activity of both enzymes. This behaviour is also exhibited by a variety of sialidases from other sources [2, 6].

The relative hydrolysis rates obtained for the substrates tested are summarized in Table 4. The natural and the recombinant sialidases from *C. septicum* show highest

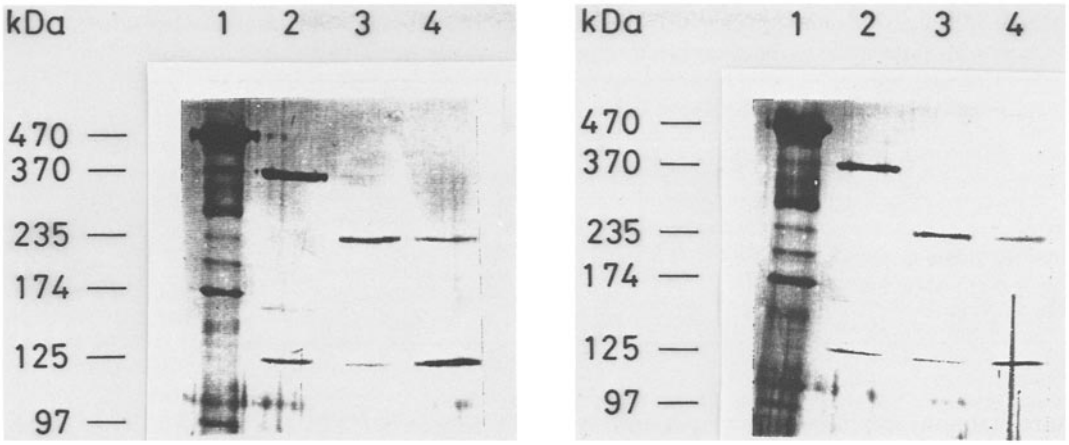

Figure 5. SDS-PAGE of (left) the recombinant and (right) the natural sialidase of *Clostridium septicum*: 1, molecular weight standard; 2, first activity peak; 3, second activity peak; 4, third activity peak.

Table 3. Influence of divalent cations, EDTA and Triton on the sialidase activity. The activity values are given in %; assays without additions are set to 100%.

Substance	*Concentration*	*Recombinant sialidase*	*Natural sialidase*
Triton X-100	1%	115	108
Ca^{2+}	50 mM	115	111
Mg^{2+}	50 mM	127	125
Mn^{2+}	50 mM	130	133
Co^{2+}	10 mM	130	136
Ni^{2+}	10 mM	129	130
Zn^{2+}	10 mM	120	120
EDTA	10 mM	87	74
EDTA/Ca^{2+}	10/50 mM	117	118

Table 4. Relative hydrolysis rates determined for the recombinant and natural sialidase of *Cl septicum* with natural substrates containing 1 mM glycosidically bound sialic acid. The value for saponified BSM obtained after 15 minutes incubation at 37 °C was set at 100%.

Sialidase substrate	*Type of linkage*	*Recombinant sialidase*	*Natural sialidase*
Saponified BSM[a]	α(2-6)	100	100
Fetuin	α(2-3)/α(2-6)	67	67
Native BSM	α(2-6)	58	39
Neu5Ac-lactose	α(2-3)	33	31
Neu5Ac-lactose	α(2-6)	21	23
Colominic acid	α(2-8)	20	20
Gangliosides + D[b]	α(2-3)/α(2-8)	22	17
Gangliosides-D	α(2-3)/α(2-8)	8	13
MU-Neu5Ac[c]		7	11

[a] Bovine submandibular mucine without *O*-acetyl substituents.
[b] Detergent (Triton X-100, 1%).
[c] A comparable value for MU-Neu5Ac was calculated from K_M/V_{max}.

cleavage rate with the α(2-6) linked sialic acids of saponified BSM, free of *O*-acetyl groups, and fetuin. These results reflect the properties of the sialidases of *C. chauvoei* [13] and *Trypanosoma brucei* [30]. The hydrolysis rates of sialyllactoses are considerably lower; however, a preference for the α(2-3) over the α(2-6) linkage was observed. This is in accordance with other sialidases, as all sialidases prefer α(2-3) bound sialic acids, with the exception of the enzymes from *Arthrobacter ureafaciens* [31] and *Streptococcus pneumoniae* [2]. It is also similar to other sialidases in that *O*-acetyl groups have a strong influence on the activity of this sialidase. *O*-Acetyl groups in the sialic acid side chain, as in BSM, reduce the hydrolysis rate by about 50%, as can be seen from the difference in activity with native and saponified BSM, respectively. (Table 4). Sialyllactose with 4-*O*-acetylated *N*-acetylneuraminic acid was resistant to sialidase action.

Based on these observations, the sialidase of *C. septicum* belongs to the second main group of sialidases, which includes enzymes preferring glycoproteins as substrates, according to a classification of von Nicolai [32], and Corfield *et al.* [2].

Michaelis–Menten kinetics were determined with four substrates as shown in Table 5, indicating that the sialidases exhibit a lower affinity and turnover rate for the synthetic substrate MU-Neu5Ac than for the natural substrates tested. The K_M values for both oligosaccharides are in the range described for other bacterial sialidases [2]. Although the K_M values for the two oligosaccharides are similar, the preference for substrates with α(2-3) linked sialic acid is indicated by a higher V_{max} value for Neu5Ac-α(2-3)-lactose. In accordance with the determined hydrolysis rates, the enzyme has a higher affinity to fetuin than to the other substrates tested.

The highest sialidase activity was obtained at pH 5.3 and 37 °C, which is similar to the properties of most other bacterial sialidases [11–13, 15, 27].

Table 5. Michaelis–Menten kinetics of the recombinant and natural sialidase of *C. septicum* with three natural and a synthetic substrate.

Sialidase substrate	*Recombinant sialidase*		*Natural sialidase*	
	K_M (*mM*)	V_{max} (*mU*)	K_M (*mM*)	V_{max} (*mU*)
Fetuin	0.55	1.00	0.65	0.95
Neu5Ac-α(2-3)-lactose	2.26	0.49	2.70	0.50
Neu5Ac-α(2-6)-lactose	2.14	0.27	2.76	0.28
MU-Neu5Ac	5.60	0.19	5.80	0.27

The temperature optimum resembles a value found for most sialidases, except the 'large', secreted isoenzyme of *C. perfringens* with an optimum at 55 °C [11].

The investigations described demonstrate that the enzymatic properties and protein pattern on SDS-PAGE of the recombinant and natural sialidases of *C. septicum* are almost identical. Slight differences such as the influence of inhibitors, the relative hydrolysis rates and the kinetic data might be caused by a trace of contaminating proteins, due to the use of enzyme solutions from the penultimate purification step as described. Thus, these similar biochemical characteristics can be considered as proof for the identity of the cloned enzyme with the natural one.

Acknowledgements

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References

1. Müller HE (1974) *Behring Inst Mitt* **55**:34–56.
2. Corfield AP, Michalski J-C, Schauer R (1981) In *Sialidases and Sialidoses* (Tettamanti G, Durand P, DiDonato S, eds) Vol 4, pp 3–70. Milano: Edi Ermes.
3. Müller HE (1982) *Bioforum* **1/2**:16–22.
4. Gabriel O, Heeb MJ, Hinrichs M (1984) In *Molecular Basis of Oral Microbial Adhesion*, pp 45–52. Philadelphia: ASM.
5. Ezepchuk YV, Vertier YV, Kostyukova NN (1973) *Biull eksp biol med* **75**:63–65.
6. Corfield AP, Schauer R (1982) In *Sialic Acids* (Schauer R, ed), pp 5–50. Vienna: Springer-Verlag.
7. Corfield AP, Lambré C, Michalski J-C, Schauer R (1992) *Conferences Philippe Laudat 1991*, pp. 111–134. Paris: INSERM.
8. Müller HE (1976) *Zbl Bact Hyg IA* **235**:106–10.
9. Schauer R (1983) In *Structural Carbohydrates in the Liver* (Popper H, Reutter W, Köttgen E, Gudat F, eds), pp 83–97. Boston: MTP Press.
10. Schauer R (1982) *Adv. Carbohydr Chem Biochem* **40**:131–234.
11. Nees S, Veh R, Schauer R (1975) *Hoppe-Seylers Z Physiol Chem* **356**:1027–42.
12. Roggentin P, Berg W, Schauer R (1987) *Glycoconjugate J* **4**:349–59.
13. Heuermann D, Roggentin P, Kleineidam RG, Schauer R (1991) *Glycoconjugate J* **8**:95–101.
14. Cato EP, George WL, Finegold M (1986) in *Bergey's Manual of Systematic Bacteriology* (Sneath PHA, Mair NS, Sharpe ME, Holt JG, eds), Vol. 2, pp 1141–200. Baltimore: Williams & Wilkins.
15. Rothe B, Rothe B, Roggentin P. Schauer R (1991) *Mol Gen Gener* **226**:190–97.
16. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning – A Laboratory Manual.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
17. Petersen GL (1977) *Anal Biochem* **83**:346–56.
18. Laemmli UK (1970) *Nature* **277**:680–85.
19. Heukeshoven J, Dernick R (1983) *Electrophoresis* **6**:103–12.
20. Potier JT, Mameli L, Bélisle M, Dallaire L, Melançon SB (1979) *Anal Biochem* **101**:287–96.
21. Veh RW, Michalski J-C, Corfield AP, Sander-Wewer M, Gies D, Schauer R (1981) *J Chromatogr* **212**:313–22.
22. Reuter G, Pfeil R, Stoll S, Schauer, R, Kamerling JP, Versluis C, Vliegenthart JFG (1983) *Eur J Biochem* **134**:139–43.
23. Schauer R (1987) *Methods Enzymol* **138**:132–61.
24. Ledeen RW, Yu RK (1982) *Methods Enzymol* **83**:139–91.
25. Warren L (1959) *J Biol Chem* **234**:1971–75.
26. Teufel M, Roggentin P, Schauer R (1989) *Biol Chem Hoppe-Seyler* **370**:435–43.
27. Hoyer LL, Roggentin P, Schauer R, Vimr ER (1991) *J Biochem (Tokyo)* **110**:462–67.
28. Air GM, Laver WG (1989) *Proteins Structure Function Genet* **6**:341–56.
29. Schauer R, Wember M (1989) *Biol Chem Hoppe-Seyler* **370**:183–90.
30. Engstler N, Reuter G, Schauer R (1992) *Mol Biochem Parasitol* **54**:21–30.
31. Uchida T, Taikada Y, Sugimori T (1979) *J Biochem (Tokyo)* **86**:1573–85.
32. Von Nicolai H (1975) Habilitation thesis, University of Bonn.