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# Purification and Characterization of Sialidase from *Clostridium sordellii* G12

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**Sialidase secreted by the urease-positive *Clostridium sordellii* strain G12 was isolated from culture medium and purified to apparent homogeneity as estimated by Fast Protein Liquid Chromatography (FPLC) and sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). For this purpose, ion-exchange chromatography, gel filtration, isoelectric focusing, and FPLC on ion-exchange resin and gel filtration materials were used. The sialidase was purified 159 300-fold from 5 l of culture medium, yielding 9 $\mu$g of enzyme protein with a specific activity of 480 U/mg. For the denatured (SDS-PAGE) and native (FPLC) sialidase relative molecular masses of 40 000 and 38 500 Da, respectively, were estimated. The substrate specificity, kinetic data, and pH-optimum of the enzyme are similar to those of other bacterial sialidases. The influences of salt or serum proteins on enzyme activity are of interest.**

Sialidases (neuraminidases; EC 3.2.1.18) are responsible for the release of sialic acids from a wide range of sialic acid-containing compounds. They are widely distributed in viruses, bacteria, protozoa, and animals [1, 2]. Although the number of identified sialidases is large, only a few have been purified from animal and microbial sources and studied in detail. Regarding clostridial species, the purification of sialidase from *Clostridium perfringens* has been described [3]. In the present study, sialidase of a *Clostridium sordellii* strain was selected for purification, due to its high activity in culture medium, solubility and low molecular weight [4]. Our aim was to obtain the pure enzyme in relatively large amounts for its characterization and for sequencing of the *N*-terminal region for genetic investigations. Furthermore, this enzyme is of great interest in gas gangrene caused by *Clostridium sordellii*, as it is believed to be of importance in the spreading of this bacterial infection. Polyclonal antibodies have been raised against this sialidase using the partially purified enzyme, with which an immunological diagnosis of clostridial infections is possible [5]. However, for this purpose polyclonal, monospecific antibodies against the pure sialidase are more desirable.

**Abbreviations:** MU-Neu5Ac, 4-methylumbelliferyl $\alpha$-D-*N*-acetylneuraminic acid; Ganglioside GD1a, IV$^3$NeuAc, II$^3$NeuAc-GgOse$_4$Cer; Neu5Ac2en, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid.

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## Materials and Methods

### *Bacterial Strain*

The *Clostridium sordellii* strain G12 (collection number of Untersuchungsinstitut I des Sanitätsdienstes der Bundeswehr, D-2300 Kronshagen) was kindly supplied by Dr. Rainer Hobrecht. This strain was selected for being non-pathogenic and non-toxic to guinea pigs and mice, respectively (R. Hobrecht, personal communication). It resembles the phenotype I of *Clostridium sordellii* characterized by the presence of sialidase, urease, arginine deaminase, and by the growth inhibiting effect of mannose [6].

### *Cultivation*

Cells were grown anaerobically at 37°C in Todd-Hewitt-broth (Difco, Detroit, MI, USA), supported by 0.1 mM sialoglycopeptides from edible bird nest substance prepared as described by Nees *et al.* [3]. After multiplication of bacteria by stepwise inoculation of 100 ml, 1 l, and 4 l, the main culture was centrifuged 5 h after the last inoculation (10 000 × *g*, 20 min, 4°C), and the supernatant was immediately used for enzyme isolation.

### *Sialidase Purification*

Enzyme activity was routinely tested with 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac), as described below. Unless stated otherwise, all purification steps were done at room temperature. The culture supernatant was diluted with an equal volume of water and adjusted to pH 6.0. The solution (10 l) was applied to a column (6 × 18 cm) containing Q-Sepharose fast flow (Pharmacia, Freiburg, W. Germany). The effluent was discarded and the column washed with 1 l of 0.02 M piperazine buffer, pH 6.0. Sialidase was eluted by a linear gradient of 0-0.3 M NaCl in the piperazine buffer (2 l). Fractions containing sialidase activity higher than in the culture medium were collected and concentrated by pressure dialysis at 4°C on ultrafilters (SM 14539, Sartorius, Göttingen, W. Germany) with a molecular weight limit of 10 000 in a 500 ml Amicon cell. The concentrate (10-20 ml) was filtered on a Sephadex G-100 column (Pharmacia) (3.5 × 95 cm) equilibrated and run (14 ml/h) with 0.1 M sodium acetate buffer, pH 5.5, at 4°C. Active fractions were collected, concentrated, dialyzed against water in the Amicon cell, and freeze-dried.

For isoelectric focusing, a granulated gel (Sephadex IEF) with the dimensions 10 × 20 × 0.2 cm containing the ampholines Servalyt 4-5 with 20% Servalyt 3-10 was prepared on a glass plate. The freeze-dried sample was mixed with a part of the gel layer (3 ml) which had been removed from the plate, and subsequently reinserted into the gel. Running conditions were 30 W for 6 h (8 000 Vh) at 3°C. The sialidase-positive zone was detected by stepwise withdrawal of 0.5 cm regions from the gel, followed by the addition of 1 ml water to each fraction and analysis of pH and enzyme activity in the supernatant, using MU-Neu5Ac as substrate. Active fractions were collected in a small column and the enzyme was eluted with water (3 gel vol) from the gel. The sialidase solution was concentrated to 2 ml and dialyzed against water on ultrafilters (SM 14529, Sartorius) with a cut off of 5 000 Da in a 10 ml Amicon cell at 4°C.

The total sample was then applied to a FPLC mono Q column (HR 5/5, Pharmacia) equilibrated with 20 mM piperazine buffer, pH 6.0, at a flow rate of 1 ml/min. The enzyme was recovered in a linear 0-0.3 M KCl gradient in this buffer. Active fractions were collected, concentrated, dialyzed against water (see above), and rechromatographed 3-4 times under the same conditions, until a single enzyme peak appeared during elution. Fractions with sialidase activity were combined and concentrated (see above) to 0.2 ml and applied to a Superose 12 column (HR 10/10, Pharmacia) equilibrated and eluted with 0.1 M sodium acetate buffer, pH 5.5, at a flow rate of 0.5 ml/min and a fraction volume of 0.5 ml. Active fractions were collected, dialyzed against water in a 10 ml Amicon cell and stored frozen at -20°C or freeze-dried.

*Assays*

During enzyme isolation, sialidase activity was assayed by the method of Potier *et al.* [7], using 0.2 mM MU-Neu5Ac as substrate in 0.1 M sodium acetate buffer, pH 5.5, in a total volume of 0.1 ml. This glycoside was synthesized as described earlier [8]. The reaction was stopped after 10 min at 37°C by the 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 (M-1000) filter-spectrofluorimeter 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 $\mu$mol substrate hydrolyzed per min.

For the investigation of substrate specificity with natural substrates, sialidase activity was tested in 0.1 M sodium acetate buffer, pH 5.5, at 37°C in a total volume of 0.1 ml with sialyllactoses or glycoconjugates as substrates, containing 1 mM bound sialic acid, as measured by the microadaptation of the orcinol/$Fe^{3+}$/HCl-reaction [9]. They included ganglioside GD1a, $\alpha_1$-acid glycoprotein from equine or human serum, fetuin (Sigma, Deisenhofen, W. Germany), colominic acid (Sigma), glycopeptides from equine or bovine submandibular gland mucin, Neu5Ac-$\alpha$(2-3)-lactose, and Neu5Ac-$\alpha$(2-6)-lactose. Ganglioside GD1a was prepared by a methanol/chloroform extraction of bovine brain, followed by DEAE ion-exchange chromatography and silica gel chromatography [10]. Equine $\alpha_1$-acid glycoprotein was isolated by precipitation with sulfosalicylic acid, followed by dialysis and chromatography on DEAE-cellulose and Sephadex G-75 [11]. Human $\alpha_1$-acid glycoprotein was purchased from Sigma. Bovine and equine submandibular gland mucins were isolated as described [12, 13].

Glycopeptides were prepared by digestion of these mucins using 0.1 mg papain (Merck, Darmstadt, W. Germany) and 0.5 mg mucin in 1 ml of 10 mM sodium phosphate buffer, pH 6.5, at 37°C for 24 h. They were purified by chromatography on DEAE-Sephacel and Sephadex G-75 [14]. Neu5Ac-$\alpha$(2-3)-lactose and Neu5Ac-$\alpha$(2-6)-lactose were isolated from bovine colostrum [15]. With these substances, the enzymatic hydrolysis reaction was followed up to 60 min by determination of free sialic acid by the periodic acid/thiobarbituric acid assay [16]. The sialic acids released from the equine glycoproteins and bovine submandibular gland glycopeptides were also analyzed by TLC after purification by ion-exchange chromatography [9].

The pure enzyme was tested to see whether its activity could be influenced by $Ca^{2+}$, EDTA, $Hg^{2+}$, sialidase inhibitors [17], the hydrolysis product Neu5Ac, and protein or salt concentrations. The assays with $Ca^{2+}$ and EDTA were carried out in total volumes of 0.1 ml, each containing 10 $\mu$l sialidase solution (16 mU/ml), 20 $\mu$l of 1 mM MU-Neu5Ac, 0, 10

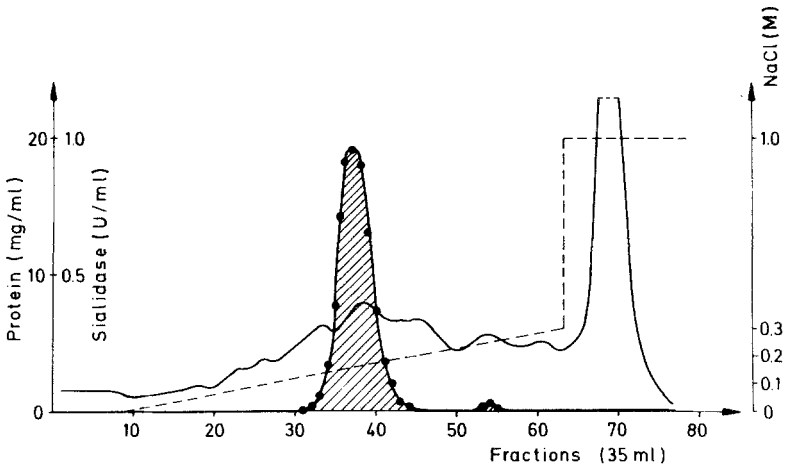


**Figure 1.** Elution profile of proteins (—) from 5 l of culture medium of *Clostridium sordellii* G12, retained by ion-exchange resin (Q-Sepharose in a 6 × 18 cm column) at pH 6.0, and eluted with a 0-0.3 M NaCl gradient (---). The hatched area represents the fraction containing sialidase activity.

or 20 µl of a 1 mM or 100 mM $CaCl_2$-solution, or 0, 10 or 20 µl of a 1 mM or 10 mM EDTA-solution, and 70, 60 or 50 µl of 0.1 M 3,3-dimethylglutaric acid buffer, pH 6.0. The assays were incubated for 15 min at 37°C with the substrate added immediately. Enzyme activity was also tested after pre-incubation with these substances for 2 h at 37°C without substrate. The influence of mercury ions on enzyme activity was tested with assays containing 10 µl of sialidase solution (38 mU/ml), 10 µl of 1 mM MU-Neu5Ac, 10 µl of $HgCl_2$ solutions (0.01, 0.1, 1 mM $Hg^{2+}$), and 70µl of 0.1 M sodium acetate buffer, pH 5.5. Fluorescence was measured after incubation for 10 min at 37°C. The influence of 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en; Boehringer, Mannheim, W. Germany), *N*-(4-nitrophenyl)oxamic acid, a gift of Dr. R.W. Veh (Bochum, W. Germany), or *N*-acetylneuraminic acid (Neu5Ac), isolated from edible bird nest substance [18], in final concentrations of 1, 0.5, 0.1 and 0.05 mM was tested with the assays (0.1 ml) including 1 µl sialidase (43 mU/ml), 20 µl of 1 mM MU-Neu5Ac solution, and 0.1 M sodium acetate buffer, pH 5.5. Incubation times were 30 min at 37°C. When measuring the influence of non-enzymic protein on sialidase activity, the assays contained 10 µl of protein solutions of various concentrations (80 mg/ml bovine serum albumin or 67 mg/ml human serum protein, 6 times stepwise 1:2 diluted with water), 10 µl sialidase solution (19 mU/ml), 10 µl of 1 mM MU-Neu5Ac solution, and 0.1 M sodium acetate buffer, pH 5.5, to 0.1 ml. The samples were incubated for 10 min at 37°C. For determination of the effect of ionic strength on enzyme activity, assays were performed containing 10 µl 0.5 M sodium acetate buffer, pH 5.5; 0, 5, 10, 20, 40, or 60 µl of 1 M NaCl solution; 60, 55, 50, 40, 20, or 0 µl of water, 10 µl sialidase (25 mU/ml), and 20 µl of 1 mM MU-Neu5Ac.

Stability of the pure sialidase to freezing and thawing was tested with 32 mU/ml sialidase in 0.1 M sodium acetate buffer, pH 5.5. After each cycle, 10 µl of this solution was measured with the MU-Neu5Ac assay.

**Table 1.** Purification of sialidase from *Clostridium sordellii* G12. The values are means from the processing of three 5 l cultures.

| Purification step | Protein | Sialidase activity | | Purification | Recovery |
|---|---|---|---|---|---|
| | (mg) | (U) | (U/mg protein) | factor | (%) |
| I. Culture medium | 89000 | 244 | 0.003 | 1 | 100 |
| II. Ion-exchange chromatography | 2800 | 218 | 0.078 | 26 | 89 |
| III. Gel filtration | 60 | 237 | 3.95 | 1317 | 97 |
| IV. Isoelectric focusing | 1.85 | 64 | 34.6 | 11532 | 26 |
| V. Ion-exchange FPLC | 0.097 | 16.8 | 173 | 57700 | 6.9 |
| VI. Gel filtration FPLC | 0.009 | 4.3 | 478 | 159300 | 1.8 |

Protein was routinely measured by absorption at 280 nm ($A_{280}$ of 1 = 1 mg protein/ml), and in the pools from sialidase enrichment steps additionally by the method of Lowry *et al.* as modified by Peterson [19].

SDS-PAGE of the sialidase fractions was performed as described by Laemmli [20], using a gradient separation gel (6-15% T, 2.7% C; 12 × 16 × 0.15 cm). Running conditions were 40-60 V for 6 h at 15°C. The reagents were purchased from BDH (Promochem, Wesel, W. Germany) and were of highest purity. Gels were stained as described [21].

## Results and Discussion

### *Sialidase Isolation*

The cultures were harvested after 5 h of bacterial growth before sialidase activity had reached its maximum in the medium (24 h), to reduce the possibility of degradation of enzyme protein. Since "fast flow" ion-exchange resins are now commercially available, the time-consuming procedures used earlier [3, 4, 22]. for the isolation of sialidases from large volumes of culture medium need no longer be applied. In addition to the rapidity of the new method (4 h for 10 l), large amounts of contaminating proteins can be removed from the sialidase in the effluent and wash, or during fractionation, as is shown in Fig. 1 and Table 1. During gel filtration, which required two days for completion, enzyme activity eluted at a position of low protein content resulting in an additional high purification (Fig. 2). Both procedures apparently do not lead to a significant loss of enzyme activity measured by the fluorimetric assay. This may be caused by substrates, e.g. sialoglycopeptides, added to the culture medium to stimulate sialidase production. They compete with the MU-Neu5Ac assay, and are gradually removed during the purification procedures. Therefore, the values obtained for the broth and the pool after ion-exchange chromatography may not represent the total sialidase activity present. No evidence for the occurrence of an inhibitor was obtained. Thus, an enzyme preparation enriched over 1000-fold can easily be obtained in high yield by the application of only two purification steps.

On isoelectric focusing for further purification, protein was reduced to 3% of the original amount, but this step also included a marked decrease of enzyme yield. As a

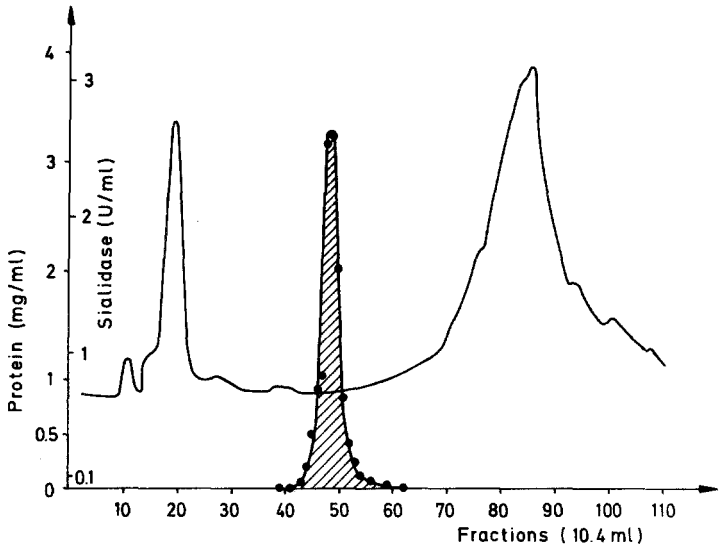


**Figure 2.** Gel filtration of the sialidase-active pool from Q-Sepharose on Sephadex G-100. The continuous line represents eluted protein; sialidase activity was found in the hatched area.

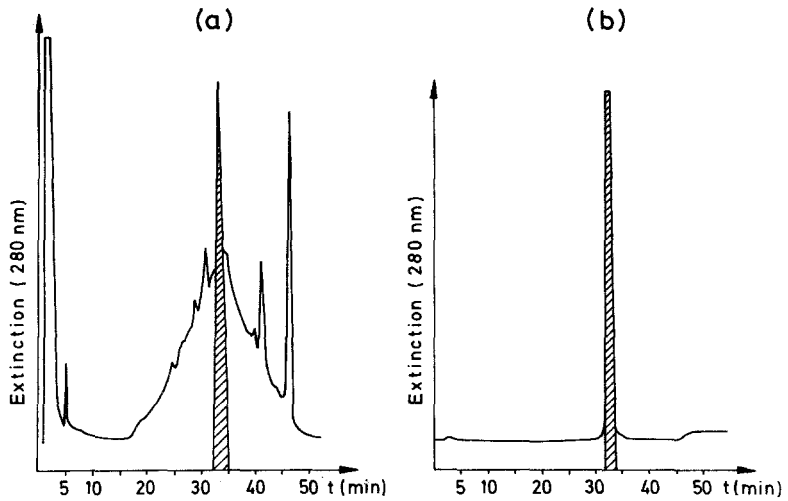


**Figure 3.** FPLC on ion-exchange resin (mono Q) of the active pool after isoelectric focusing. a) Proteins retained at pH 6.0 were eluted (—) with a 0-0.3 M KCl gradient. b) Rechromatography of the active fractions, after three cycles, resulted in a single peak, representing the active protein (hatched area).

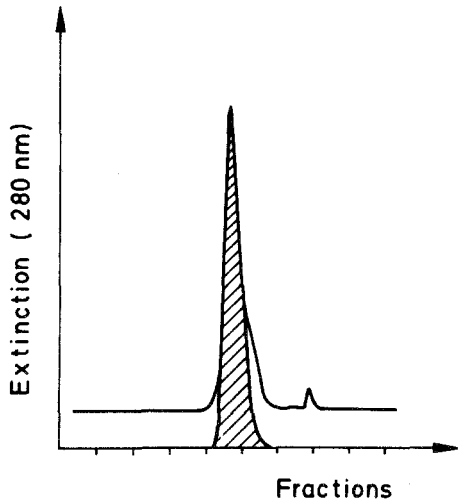


**Figure 4.** FPLC-gel filtration (Superose 12) of the active pool after ion-exchange FPLC. Sialidase activity (hatched area) shows coincidence with the main protein peak. A few minor protein contaminants are, however, visible.

further result of this purification step the isoelectric point of the enzyme was determined to be pH 4.4. Three or four cycles of FPLC ion-exchange chromatography of the enriched sialidase led to the elution of a single peak containing enzyme of high specific activity (Fig. 3a/3b). As amino acid sequencing (not described here) from the *N*-terminus of the protein obtained from this purification step revealed a small amount of contaminating protein, which slightly interfered with this analysis, the enzyme was further purified by FPLC gel filtration. Fig. 4 shows that contaminating proteins could be separated from the sialidase protein, leading to a further increase in specific activity. The efficiency of the latter step resulted in a single protein band of the sialidase on SDS-PAGE (Fig. 5).

The values for protein and sialidase activity of each purification step are summarized in Table 1, representing the average values obtained for the processing of three 5 l cultures. Although the presented values were routinely obtained, in some other isolation procedures under identical conditions maximum values of 1330 U per mg protein or 6% yields were reached. The specific activity of the pure *C. sordellii* enzyme is in the range described for the *C. perfringens* sialidase by Nees *et al.* [3].

*Sialidase Properties*

The molecular weight measured by gel filtration and SDS-PAGE, is 38 500 and 40 000, respectively. Thus, the enzyme does not consists of subunits. The pure sialidase of *C. sordellii* G12 is active over a broad pH range, from 4.5-8.5 with an optimum at pH 6.0. A similar broad activity range was found for its dependence on temperature. The enzyme is active from 4°C to 55°C with activity at these extreme temperatures still 10% of its maximum at 36°C. These values may indicate that the enzyme is not only produced by the bacteria to attack glycoconjugates of homiothermic vertebrate hosts, but also to decompose organic material at various biotopes.

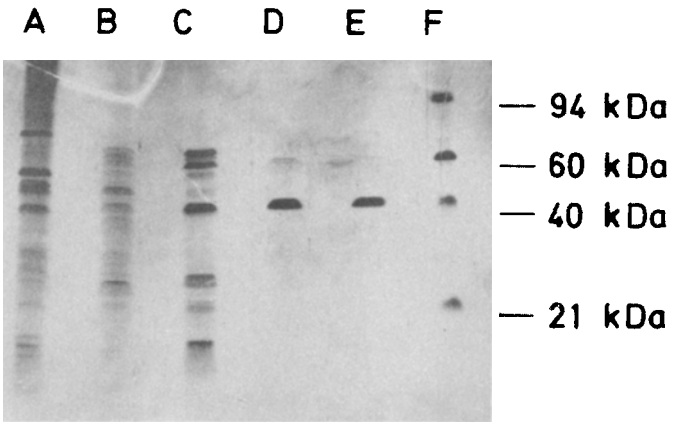


**Figure 5.** SDS-PAGE of samples obtained from the individual purification steps of *Clostridium sordellii* G12 sialidase. The lanes contain samples after: A, ion-exchange chromatography; B, gel filtration; C, isoelectric focusing; D, ion-exchange-FPLC (3 cycles); E, FPLC-gel filtration. Lane F contains the molecular weight standards, from top to bottom, phosphorylase B, catalase, aldolase (rabbit muscle), and soybean trypsin inhibitor. Protein bands became visible after silver staining.

In addition to MU-Neu5Ac, the sialic acid glycosidic bonds of the natural substrates tested are hydrolyzed by the pure sialidase, except 4-*O*-acetylated sialic acids. The resistance of *N*-acetyl-4-*O*-acetylneuraminic acid was proven with glycopeptides from equine submandibular gland mucin and $\alpha_1$-acid glycoprotein from equine serum. It was shown by TLC that the small amounts (see below) of sialic acids released from these compounds are not *O*-acetylated. This behaviour towards 4-*O*-acetylated sialic acids corresponds to that observed with other bacterial and mammalian sialidases [1, 2, 23]. In contrast, 9-*O*-acetylated sialic acids can be liberated from bovine submandibular gland glycopeptides by the clostridial sialiadase. The substrates studied are given in order of decreasing hydrolysis rates, as follows: Neu5Ac-$\alpha$(2-3)-lactose (100%), $\alpha_1$-acid glycoprotein from human serum (37.6%), Neu5Ac-$\alpha$(2-6)-lactose (31.6%), colominic acid (31.6%), fetuin (27%), glycopeptides from bovine submandibular gland mucin (8.6%), ganglioside GD1a in the presence of 1% Triton CF 54 (8.5%), $\alpha_1$-acid glycoprotein from equine serum (5.3%), glycopeptides from equine submandibular gland mucin (2.6%), and ganglioside GD1a without Triton (2.2%).

The values indicate that small and easily accessible substrates like sialyllactoses, the sialic acid polymer, and serum glycoproteins are rapidly hydrolyzed by the *C. sordellii* sialidase, as is the case for most other sialidases [1, 2, 17]. In general, substrates containing $\alpha$(2-3)-linked sialic acid are better substrates than those in $\alpha$(2-6) or $\alpha$(2-8)-linkage. This behaviour was observed here and in all mammalian, trypanosome, viral and most bacterial sialidases studied so far [1, 2, 24, 25]. In contrast to the previously described sialidases, no difference in the hydrolysis rate was found between $\alpha$(2-6) and $\alpha$(2-8)-linkages (compare Neu5Ac-$\alpha$(2-6)-lactose and colominic acid). The marked reduction in activity with gangliosides may be due to their micellar structure. This effect could be reduced by the addition of Triton. The relatively low susceptibility of mucin sialic acids from mucin glycopeptides for the clostridial enzyme is a common property of sialidases, too [1, 2].

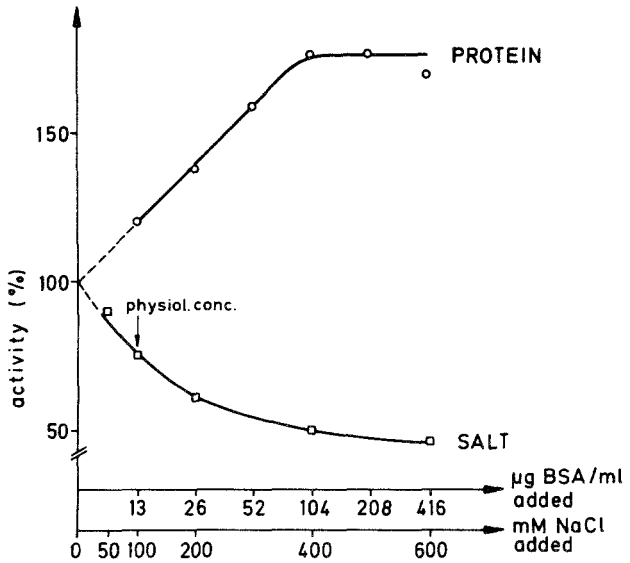


**Figure 6.** Dependence of sialidase activity in 50 mM sodium acetate buffer, pH 5.5, on protein (bovine serum albumin) or NaCl concentrations. For experimental conditions see Methods.

The highest hydrolysis rate was obtained with a 1 mM solution of the synthetic substrate MU-Neu5Ac (160%; Neu5Ac-α(2-3)-lactose = 100%), in accordance with a variety of other sialidases [2, 17]. For this reaction a $K_M$ of 0.5 mM and a catalytic activity of 1000 MU-Neu5Ac molecules/sec hydrolyzed by one enzyme molecule was determined.

Mercury ions completely inhibit enzyme activity even at 0.01 mM $HgCl_2$, indicating the presence of essential SH groups. This effect has been found with all sialidases tested [1, 2]. In the case of human leucocyte sialidase, however, 1 mM $Hg^{2+}$ led to only 76% inhibition [17]. The sialidase from *C. sordellii* is inhibited by Neu5Ac2en, *N*-(4-nitrophenyl)oxamic acid and the enzymatic hydrolysis product Neu5Ac in decreasing order, as is known for a variety of sialidases from other sources [1, 2, 17]. Thus, 50% of sialidase activity was inhibited by 0.05 mM Neu5Ac2en, or 0.37 mM *N*-(4-nitrophenyl)oxamic acid. Neu5Ac has only a slight inhibitory effect, since only 10% inhibition was observed at 0.7 mM concentration.

*C. sordellii* sialidase activity was not increased by the addition of $Ca^{2+}$, known to be promotive for the activity of e.g. *Vibrio cholerae* [2] or human [1, 17] sialidases. Accordingly, no effect was detectable after the addition of EDTA to the sialidase assay. On the other hand, the activity of the *C. sordellii* enzyme is very sensitive to an increase in ionic strength. For instance, the increase of sodium chloride concentration (Fig. 6) diminished the activity to 45% at 600 mM NaCl. Such an effect is also known for lysosomal sialidases [1]. The opposite effect on enzyme activity was observed by the addition of proteins, which led to a marked increase of enzyme activity (Fig. 6). This effect was independent of the nature of the proteins tested, as comparable results were obtained with bovine serum albumin and human serum. Activation, however, depends on the

quantity of protein added, maximum enzyme activity being reached in a solution of 100 $\mu$g protein per ml. It is suspected that protein or salt changes the conformation of the enzyme protein thereby influencing the active center of the sialidase, as was also described for lysosomal sialidases [1].

The enzyme was stable to freezing and thawing. No loss of activity was measured after five cycles, in complete contrast to the labile human sialidases [1, 2, 17].

The sialidase of *C. sordellii* may be of wide interest, since apathogenic strains of this species are available, which secrete large amounts of enzyme within a few hours. The enzyme can easily be separated from most contaminating proteins due to its low molecular weight and relatively acidic isoelectric point. After a few further chromatographic steps a pure enzyme protein with broad substrate specificity can be obtained, which may be useful in the fields of cell biology, immunology and molecular biology.

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**References**

1 Corfield AP, Michalski J-C, Schauer R (1981) in Sialidases and Sialidoses, eds. Tettamanti G, Durand P, DiDonato S, Edi Ermes, Milan, 4:3-70.
2 Schauer R (ed.) (1982) Sialic Acids - Chemistry, Metabolism and Function, Cell Biol Monogr, Vol 10, Springer, Wien.
3 Nees S, Veh RW, Schauer R (1975) Hoppe Seylers Z Physiol Chem 356:1027-42.
4 Gutschker-Gdaniec G, Sander-Wewer M, Roggentin P, Hobrecht R, Schauer R (1983) in Proc 7th Int Symp Glycoconjugates, eds. Chester MA, Heinegård D, Lundblad A, Svensson S, Secretariat, Lund, p 450-51.
5 Sander-Wewer M, Gutschker-Gdaniec G, Kietzmann E, Roggentin P, Hobrecht R, Schauer R (1983) in Proc 7th Int Symp Glycoconjugates, eds. Chester MA, Heinegård D, Lundblad A, Svensson S, Secretariat, Lund, p 448-49.
6 Roggentin P, Gutschker-Gdaniec G, Schauer R, Hobrecht R (1985) Zentralbl Bakteriol Mikrobiol Hyg [A] 260:319-28.
7 Potier M, Mameli L, Bélisle M, Dallaire L, Melancon SB (1979) Anal Biochem 94: 287-96.
8 Berg W, Gutschker-Gdaniec G, Schauer R (1985) Anal Biochem 145:339-42.
9 Schauer R (1987) Methods Enzymol 138:132-61.
10 Ledeen RW, Yu RK (1982) Methods Enzymol 83:139-91.
11 Sander M, Veh RW, Schauer R (1979) in Proc 5th Int Symp Glycoconjugates, eds. Schauer R, Boer P, Buddecke E, Kramer MF, Vliegenthart JFG, Wiegandt H, Thieme, Stuttgart, p 358-59.
12 Tettamanti G, Pigman W (1968) Arch Biochem Biophys 124:41-50.
13 Huser H, Módy E, Faillard H (1973) Hoppe Seylers Z Physiol Chem 354:749-59.
14 Corfield AP, Parker TL, Schauer R (1979) Anal Biochem 100:221-32.

15 Veh RW, Michalski J-C, Corfield AP, Sander-Wewer M, Gies D, Schauer R (1981) J Chromatogr 212:313-22.
16 Warren L (1959) J Biol Chem 234:1971-75.
17 Schauer R, Wember M, Tschesche H (1984) Hoppe Seylers Z Physiol Chem 365:419-26.
18 Schauer R, Corfield AP (1982) in Sialic Acids - Chemistry, Metabolism and Function, ed. Schauer R, Cell Biol Monogr Vol 10, Springer, Wien, p 51-57.
19 Peterson GL (1977) Anal Biochem 83:346-56.
20 Laemmli UK (1970) Nature 227:680-85.
21 Heukeshoven J, Dernick R (1985) Electrophoresis 6:103-12.
22 Schauer R, Sander-Wewer M, Gutschker-Gdaniec G, Roggentin P, Randow EA, Hobrecht R (1985) Clin Chim Acta 146:119-27.
23 Corfield AP, Sander-Wewer M, Veh RW, Wember M, Schauer R (1986) Biol Chem Hoppe Seyler 367:433-39.
24 Reuter G, Pereira MEA, Schauer R (1984) in Abstr 12th Int Carbohydr Symp, eds. Vliegenthart JFG, Kamerling JP, Veldink GA, Vonk, Utrecht, p 311.
25 Michalski J-C, Corfield AP, Schauer R (1986) Biol Chem Hoppe Seyler 367:715-22.