Glycoconjugate J (1985) 2:45—60

The Interaction of *Clostridium perfringens* Sialidase with Immobilized Sialic Acids and Sialyl-Glycoconjugates*

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Received October 29, 1984.

Key words: bacterial and viral sialidases, affinity chromatography, sialic acid ligands, sialyl-glycoconjugate ligands, hydrophobic ligands

Clostridium perfringens **sialidase is adsorbed by sialic acid immobilized on adipic acid dihydrazido-Sepharose 4B and/or polymethylacrylic hydrazido-Sepharose 4B, through its carboxyl group, C-7 to C-9 side chain, or its amino function as D-neuraminic acid-β-methyl glycoside or 2-deoxy-2,3-didehydroneuraminic acid. Sialidase binding was strongest to the amino-linked adsorbents, but purification was low and the enzyme could not be eluted with substrate or free sialic acid. Low binding of the sialidase to the non-substituted, blocked supports suggested that hydrophobic interactions were involved, and this was confirmed by adsorption of the enzyme on alkyl agaroses with approximately 80% of total sialidase adsorbed on decyl-agarose. Genuine affinity chromatography of sialidases is possible on immobilized sialyl-glycoconjugates, and *C. perfringens* sialidase could be purified to the same specific activity as the electrophoretically homogeneous enzyme using submandibular gland mucus glycoprotein adsorbents. Sialidases from *Vibrio cholerae, Arthrobacter ureafaciens*, Newcastle disease virus, Fowl plague virus and Influenza A_2 virus also bound to immobilized sialic acids and sialyl-glycoconjugates.**

The sialidases (*N*-acylneuraminosyl glycohydrolases; EC 3.2.1.18) occur in a wide range of microorganisms and animals [1]. Purification of microorganism sialidases with high specific activity has allowed their use as analytical tools in glycoconjugate biochemistry.

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*Dedicated to Prof. Dr. Hans Faillard on the occasion of his 60th birthday.

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Animal sialidases have low specific enzyme activities and are predominantly membrane bound [1]. Many of the sialidase activities reported in the literature have not been purified [1]. This is particularly so for animal enzymes where lability, low concentration and inefficient solubilization from membranes have hampered progress. The availability of a rapid affinity technique for general use would be a great asset to sialidase research.

Immobilized ligands have been used in the purification of both microorganism and animal sialidases. Sialyl-glycoconjugates [2-5], sialic acid and analogues, and inhibitors having no structural relationship to sialic acid [5,8,9], have been immobilized with the aim of sialidase purification, however, with varying success. In order to assess the value and nature of sialidase interaction with immobilized ligands we have prepared and tested immobilized sialic acid, sialic acid analogues, sialyl-glycoconjugates, lectins and hydrophobic ligands with partially purified *Clostridium perfringens* sialidase.

Materials

The following materials were obtained from the suppliers indicated: Sepharose 4B and Heparin-Sepharose CL-6B from Pharmacia (Uppsala, Sweden); Concanavalin A (Con A) and Fetuin from Sigma Chemical Co (St. Louis, Mo, USA); Ovomucoid (trypsin inhibitor) from Boehringer Mannheim (W. Germany); Colominic acid from Koch-Light (Colnbrook, UK); 2,4,6-trinitrobenzene sulphonate from Eastman Kodak (Rochester, NY, USA); Wheat germ agglutinin (WGA) from Makor Chemicals (Jerusalem, Israel); alkyl-agaroses (Cn=2-10) from Miles-Yeda (Israel) obtained via Miles, UK. Methylacrylate, thioglycollic acid, hydrazine hydrate, sodium cyanoborohydride, cyanogen bromide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI) were from Merck, (Darmstadt, W Germany). *N*-Acetyl-D-neuraminic acid (Neu5Ac) and sialyl-α(2-6)-hexasaccharide (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc), both from human urine, were a gift from Dr. J.-C. Michalski (Lille). D-Neuraminic acid-β-methyl glycoside (Neu-β-Me) was prepared as described by Schauer and Buscher [10]. A mixture of the C-7- and C-8-aldehyde derivatives of Neu5Ac was prepared and purified according to Veh *et al.* [11], omitting the borohydride reduction step. Synthesis of 2-deoxy-2,3-di-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) and its free amine (Neu2en) has been described elsewhere [12]; the compounds were a gift from Dr. J.-M Beau (Orléans). 4-Nitrophenyl oxamic acid was a gift from Dr. R.W. Veh (Bochum). Salivary mucin glycoproteins were prepared from bovine [13], porcine [14] and equine [15] submandibular glands. Glycopeptides were prepared from bovine mucin as described elsewhere [16]. *N*-Acetylneuraminyl-α(2-3)-lactose was purified as described before [17]. *C. perfringens* sialidase purified as far as the Sephadex G-75 step [18] had an activity of 0.2 U/mg protein. *Vibrio cholerae* was obtained from Behringwerke (Marburg, W Germany), *A. ureafaciens* sialidase from Nakarai Chemical Co, (Kyoto, Japan). Viral suspensions were obtained from Prof. R. Rott, Universität Giessen, and were used as sialidase enzyme in particle form [19].

Methods

Preparation of Supports and Immobilized Ligands

Polymethylacrylate, polyacrylic hydrazide and polyacrylic hydrazido-Sepharose 4B (PAH-Sepharose) were prepared as described by Wilchek and Miron [20]. Adipic acid dihydrazido-Sepharose 4B (AD-Sepharose) was synthesized as before using periodate activation of the Sepharose [16]. 4-Nitrophenyl oxamic acid was immobilized on AD-Sepharose by the method of Brossmer *et al.* [21]. A colominic acid-starch copolymer was prepared as described by Matsumoto and Osawa [22]. Sialyl-hexasaccharide was immobilized on AD-Sepharose by reaction with the support in the presence of $NaBH_3CN$ at pH 6 [16]. Con A, WGA, sialyl-hexasaccharide, fetuin, α_1-acid glycoprotein, ovomucoid, mucus glycoproteins from bovine, porcine, or equine submandibular glands, and bovine submandibular gland glycopeptides were immobilized on Sepharose 4B by the cyanogen bromide method [16].

Sialic acid and analogues were immobilized on AD-Sepharose and PAH-Sepharose using three methods:
a) *Carbodiimide (Neu5Ac).* A solution containing e.g. Neu5Ac (20 mg) and EDCI (13 mg) in 20 ml distilled water was adjusted to pH 4.7 with 0.01 M NaOH and mixed with 20 ml (wet volume) of AD- or PAH-Sepharose. The pH was maintained at 4.7 with 0.1 M HCl during incubation at 37°C for 4 h. The gel was then washed and blocked as described below.

b) *Cyanoborohydride Reduction of Schiff's Base Linkage (C-7- and C-8-aldehyde Analogues of Neu5Ac).* Sialic acid aldehyde mixture (20 mg) was dissolved in 0.1 M sodium acetate buffer, pH 6.0, containing 0.5 mM sodium cyanoborohydride (20 ml) and mixed with 20 ml (wet volume) of AD- or PAH-Sepharose. The suspension was maintained at 4°C for 48 h with occasional gentle agitation. The washing and blocking steps were completed as below.

c) *Activation of Acyl-Hydrazido to Acyl-Azido Groups (Neu-β-Me and Neu2en).* AD- or PAH-Sepharose (40 ml) was activated with HNO_2 and washed as described before [16]. The azido-Sepharose was mixed with e.g. Neu-β-Me (50 mg) in 30 ml 0.2 M $NaHCO_3$, pH 9.5, in an ice bath. The pH was maintained at 9.5 with NaOH until no further pH-deviation occurred. The gel was washed and blocked as below.

All immobilized ligands and supports were prepared in at least two separate experiments where possible to allow comparison of preparation methodology and reproducibility of ligand immobilization.

Washing of Substituted Gels

Gel samples were washed using the same procedure during preparation and after use [16], by 10 volumes each of distilled water, 2 M NaCl and distilled water, unless otherwise indicated.

Blocking Reaction

Cyanogen bromide-activated and azido supports were blocked with 0.1 M Tris-HCl as described before [16]. AD- and PAH-Sepharose supports used to immobilize sialic acid and derivatives were blocked using acetic anhydride by the method of Baues and Gray [23]. The efficiency of blocking was followed using the 2,4,6-trinitrobenzene sulphonate test [24] with 200 μl packed gel in a total assay volume of 1 ml.

Quantification of Ligand Immobilization

The measurement of bound ligand was achieved by the difference of ligand concentrations in the supernatant before and after immobilization and by direct quantification of immobilized ligand. Ligands containing sialic acid were quantified as before [16]. Immobilization of sialic acid and derivatives was measured by micro-adaptations of the periodate/thiobarbituric acid and orcinol/Fe^{3+}/HCl assays [25] of ligand concentration in the supernatant before and after immobilization. Con A, WGA and 4-nitrophenyl oxamic acid immobilization was followed by photometric measurement of supernatant samples at 280 nm.

Storage of Gels

Gels were stored in water or 2 M NaCl containing 0.02% NaN_3 at 4°C as a slurry of 2 volumes of gel to one volume of liquid.

Interaction of Sialidases with Immobilized Ligands

The interaction of sialidases with the immobilized ligands was evaluated using two methods:

a) *Batch Method.* Sialidase (25 mU) was mixed with 1.2 ml of the buffer under study (see below) and 0.5 ml gel suspension was added. The suspension was mixed and allowed to stand for 30 min, equilibrated at 20°C or 4°C. Samples (2 × 25 μl) were taken from the original buffer-sialidase solution and the gel supernatant for measurement of activity. The volume of the gel was measured directly in each incubation [16].

b) *Column Method.* Approximately 1 ml wet volume (2.5 cm × 0.7 cm) of gel was poured into glass columns having a glass sinter and washed with 10 ml of 0.05 M sodium acetate buffer, pH 4.0 (sialic acid adsorbents) or pH 5-6 (sialyl-glycoconjugate adsorbents). *C. perfringens* sialidase (~25 mU) was applied to the column in 5 ml of the same buffer and washed with 6 ml of this buffer. Elution was with 10 ml of 0.05 M sodium phosphate buffer, pH 6.5, containing 0.2 M NaCl (sialic acid adsorbents) or 10 ml 0.05 M sodium acetate buffer, pH 6.0, containing 1 M NaCl (sialyl-glycoconjugate adsorbents).

The pH and salt concentrations of several buffer systems were studied for both binding and elution of the gels. Aliquots of the sialidase solution applied to each column and from the washing and elution samples were taken and enzymic activity determined. All experiments were performed at 4°C unless indicated otherwise.

Sialidases from *V. cholerae* (~25 mU) and bound to virus particles (Newcastle disease virus, Fowl plague virus and Influenza A_2 virus) (~20 mU) were tested on columns under the same conditions as for *C. perfringens* sialidase and assayed [19] after adjusting pH and ionic strength as described below.

Binding of Sialidase to Other Immobilized Ligands

The interaction of sialidase with immobilized Con A, WGA and heparin was studied under standard conditions for these adsorbents [26], as were 4-nitrophenyl oxamic acid-AD-Sepharose [21] and colominic acid-starch gel [27].

Hydrophobic Agarose Gels

Hydrophobic agarose gels (~1 ml) with alkyl chains from n=2 to n=10 were prewashed with 0.1 M sodium acetate buffer, pH 4.5 or 5.0. Sialidase (20-40 mU) in 2 ml of the same buffer was applied to the column and eluted either with 0.1 M sodium acetate buffer, pH 4.5 or 5.0, containing 0.1 M NaCl; with 0.1 M sodium acetate buffer, pH 6.0; or with 1 M NaCl. Aliquots were removed for enzymic assay.

Enzyme and Chemical Assays

Sialidase activity was measured using *N*-acetylneuraminyl-α(2-3)-lactose as substrate as described elsewhere [19]. One unit of activity (U) is taken as 1 μmole of sialic acid released per minute at 37°C. Aliquots from column chromatography containing high salt concentration, enzyme substrate or inhibitors of sialidase activity were dialysed against 100 vol of incubation buffer for 2 h at 4°C with 3 changes. A sample of the sialidase preparation applied to the column was also dialysed at a similar dilution as a control before assay.

The measurement of protease, β-galactosidase, *N*-acetyl-β-glucosaminidase and *N*-acetyl-β-galactosaminidase activities, and protein concentration were as described elsewhere [18]. Sialic acid was measured by the periodic acid/thiobarbituric acid and orcinol/Fe^{3+}/HCl methods in a microscale [25].

Electrophoresis

Discontinuous electrophoresis on an analytical scale was as described before [28]. Gels were stained for protein with amido black [18]. Sialidase activity was localized in unstained gels after extraction of 0.5 cm sections with 200 μl 0.1 M sodium acetate buffer, pH 5.1 for 30 min at 4°C and incubation of supernatant aliquots as described above. Preparative continuous polyacrylamide gel electrophoresis was carried out as described before [18].

Table 1. Immobilized ligands and *Clostridium perfringens* sialidase bidning. The immobilization technique and measurement of ligand concentration are given under

No.	Ligand	Immobilization Technique	Support	Ligand[a] μmole/ml gel	No. of batches tested	Sialidase[b] Binding %
Sialic Acid and Derivatives						
1	*N*-Acetylneuraminic Acid (Neu5Ac)	Carbodiimide	AD-Sepharose PAH-Sepharose	1.3 1.9	2 2	80 60
2	C-7/C-8 Analogues of Neu5Ac	Schiff's base-$NaBH_3CN$	AD-Sepharose PAH-Sepharose	1.5 4.0	2 2	100 40
3	Neuraminic acid-β-methyl-glycoside (Neu-β-Me)	Acyl azide	AD-Sepharhose PAH-Sepharose	1.5 5.0 (2.5-11.0)	2 3	100 100
4	2-Deoxy-2,3-didehydro-neuraminic acid (Neu2en)	Acyl azide	PAH-Sepharose	5.0	2	100
Hydrophobic Type						
5	Alkanes C_2-C_{10}	CNBr	Agarose	15-20	1	$C_{10} >> C_2$[b]
6	4-Nitrophenyloxamic acid	Carbodiimide	AD-Sepharose	1.4	1	<5
Sialyl-Glycoconjugates						
7	Porcine submandibular gland glycoprotein	CNBr	Sepharose 4B	0.2 (0.2-0.8)	3	100
8	Bovine submandibular gland glycoprotein	CNBr	Sepharose 4B	1.0 (0.1-3.5)	4	100
9	Bovine submandibular gland glycopeptide	CNBr	Sepharose 4B	1.8	1	100
10	Equine submandibular gland glycoprotein	CNBr	Sepharose 4B	0.2 (0.1-0.6)	4	100
11	Fetuin	CNBr	Sepharose 4B	0.5 (0.2-1.2)	4	100[b]
12	α_1-Acid glycoprotein	CNBr	Sepharose 4B	0.7 (0.1-1.5)	3	100[b]
13	Ovomucoid	CNBr	Sepharose 4B	0.2	2	100
14	Sialylhexasaccharide	Schiff's base-$NaBH_3CN$	AD-Sepharose	0.7	1	15
15	Colominic acid	Epichlorhydrin	Starch gel	320.0	2	20
Others						
16	Wheat germ agglutinin	CNBr	Sepharose 4B	1.7	2	0
17	Concanavalin A	CNBr	Sepharose 4B	4.7	1	0
18	Heparin	CNBr	Sepharose CL-6B	?	1	0

[a] The concentration of immobilized ligand is expressed as μmole sialic acid/ml gel or μmole ligand/ml gel where no sialic acid is present. Ligands 16 and 17 are mg ligand/ml gel. Figures in brackets indicate the range of immobilized ligand concentrations prepared and tested.

[b] Sialidase activity adsorbed is expressed as a percentage of the total activity (25 mU) applied to a 1 ml column of adsorbent. In the case of fetuin and α_1-acid glycoprotein 100% binding of sialidase was achieved at pH 5.0. For binding by ligands 5 compare Fig. 3.

methods. Results for sialidase were obtained using 25 mU of enzyme and 1 ml of packed gel in small columns and eluting under conditions detailed under Methods.

Capacity[c] mU/ml gel	Purification Factor	Contaminating Enzyme Activity (% Remaining) Protease	β-Galactosidase	N-Acetyl-β-Glucosaminidase	Other Sialidases[d] Binding to Adsorbent
NT[e]	—	—	—	—	—
NT	—	—	—	—	—
260	1.6	<0.1	8	14	Virus, *V. cholerae*
NT	NT	—	—	—	—
320	2.5	<0.1	10	14	Virus, *V. cholerae*
1090	3.1	<0.1	8	12	Virus, *V. cholerae*
1150	3.0	<0.1	6	8	Virus, *V. cholerae*
NT	NT	—	—	—	*V. cholerae* *A. ureafaciens*
NT	NT	—	—	—	*V. cholerae*
310	25	0	<0.1	<0.1	Virus
570	32	0	<0.1	<0.1	Virus, *V. cholerae*
510	30	0	<0.1	<0.1	NT
305	34	0	<0.1	<0.1	Virus, *V. cholerae*
300	20	0	0.5	0.1	Virus, *V. cholerae*
270	21	0	1.0	0.5	Virus, *V. cholerae*
290	6	<0.1	0.9	52.0	Virus
NT	NT	—	—	—	—
NT	NT	—	—	—	*A. ureafaciens*
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	—

[c] The capacity of each column was measured by continued applications of 25 mU aliquots of C. perfringens sialidase under optimal binding conditions, until activity was detected in the effluent.

[d] Other sialidases tested which gave quantitative (100%) binding of 25 mU enzyme activity to the adsorbent under the same conditions described for *C. perfringens* sialidase.

[e] NT = not tested.

Results and Discussion

Immobilization of Ligands

The design of affinity supports for enzyme purification ideally requires an inhibitor or ligand with high affinity for the enzyme, immobilized in such a way that no additional ionic or hydrophobic interactions arise due to the linkage of the ligand to the support. In the case of the sialidases, different enzymes from many organisms are known [1] and an affinity adsorbent for general use would be a great aid to the study of these enzymes.

The ligands chosen to investigate sialidase affinity chromatography are listed in Table 1. Numbers 1 to 4 are sialic acid and derivatives immobilized on AD- and PAH-Sepharose, which were chosen as supports because of their high ligand capacity, stability and non-changed form after the immobilization step [20,29]. The blocking of unreacted amino groups on AD- and PAH-Sepharose after ligand immobilization is important for sialidase chromatography, as ionic [30] and other non-specific interactions [31] have been noted between sialidases and non-substituted supports. The binding of *C. perfringens* sialidase to non-blocked AD- and PAH-Sepharose could be shown and this was reduced after the incomplete blocking reaction with Tris and largely but not completely eliminated by acetylation [23] as shown in Table 2.

Immobilization of sialyl-glycoconjugates on Sepharose 4B as described before [16] showed no residual binding of *C. perfringens* sialidase to the support alone or its blocked form (Table 2). Such adsorbents have been proposed as genuine affinity matrices for bacterial [2,3,5,32,33], viral [4,5] and animal [5,34,35] sialidases. The affinity nature of this type of adsorbent could be confirmed in studies with sialic acid glycosides immobilized on Sepharose [6,7]. All of these adsorbents were substrates for sialidases and degradation occurs with use. Furthermore, on sialyl-glycoconjugate adsorbents subterminal monosaccharides may be exposed and thus bind other glycosidases while reducing the capacity for sialidase. Under the conditions outlined in Methods it was possible to vary the concentration of immobilized ligand [29], and several ligands were tested at different densities as shown in Table 1.

Interaction of Sialidase with Immobilized Sialic Acid and Derivatives

All of the immobilized ligands in this category bound *C. perfringens* sialidase to a significant degree (Table 1, ligands 1 to 4). Batch binding experiments indicated that pH 4 was the optimal condition for the binding of *C. perfringens* and Newcastle disease virus sialidases to these adsorbents. The use of 1 ml columns confirmed this result, and at pH values of 5 and above less than 50% of the enzyme was bound.

No quantitative binding of *C. perfringens* sialidase to carboxyl-linked sialic acid could be demonstrated, although binding to the AD-Sepharose adsorbent was higher than to the PAH-Sepharose (Table 1). *V. cholerae* sialidase did not bind at all to sialic acid linked to Sepharose through its carboxyl group [6], which is in agreement with the requirement of the free carboxyl group in enzyme-substrate complex formation [1,36,37].

Immobilization of sialic acid through the C-7 to C-8 side chain resulted in quantitative binding of *C. perfringens* sialidase to the AD-Sepharose adsorbent but only 40% to the

Table 2. Interaction of *Clostridium perfringens* sialidase with blocked and non-blocked supports. The support (1 ml packed gel) was tested with 25 mU sialidase under optimal binding conditions (pH 4) for sialic acid adsorbents (see Methods). Results are expressed as a % of the sialidase remaining bound to the column after application and wash with 10 ml of 0.05 M sodium acetate buffer pH 4.0. Blocking reactions and 2,4,6-trinitrobenzene sulphonate assay are given in the Methods.

Support	Blocking Reaction	2,4,6-Trinitrobenzene Sulphonate Test	*C. perfringens* Sialidase Binding at pH 4 (%)
Sepharose 4B	none	colourless	0
Sepharose 4B-CNBr-activated	Tris	colourless	0
AD-Sepharose	none	red	80
AD-Sepharose	Tris	light pink	40
AD-Sepharose	acetic anhydride	colourless	30
Neu-β-Me-AD-Sepharose	acetic anhydride	light yellow	100
PAH-Sepharose	none	dark red	95
PAH-Sepharose	Tris	light red	25
PAH-Sepharose	acetic anhydride	colourless	20
Neu-β-Me-PAH-Sepharose	acetic anhydride	light yellow	100

PAH-Sepharose matrix (Table 1). The length of the polymer support-ligand spacer may be significant. AD-Sepharose has the structure: polymer-NHNHCO$(CH_2)_4$CONH-ligand, while PAH-Sepharose has a very short spacer: (polymer-$CH_2)_2$-CH-CONH-ligand. The shortening of the sialic acid side chain is known to reduce sialidase activity for substrates containing these analogues [38], and the C-7 analogue of sialic acid immobilized as its glycoside on Sepharose was shown to have reduced affinity for *V. cholerae* sialidase relative to the intact sialic acid [6,7]. Thus, the results from sialidase interaction with carboxyl- and side chain-linked sialic acid adsorbents suggest that binding other than the affinity for the ligand itself plays a significant part.

Immobilization of Neu-β-Me and Neu2en through their free amino groups provided the most effective adsorbents in this group. Quantitative binding of *C. perfringens*, Newcastle disease virus, Influenza A_2 virus and Fowl plague virus sialidases could be obtained at pH 4. Several differences in the elution behaviour of *C. perfringens* sialidase indicated the participation of hydrophobic-type binding. Sialidase bound to Neu-β-Me-AD-Sepharose was eluted by 0.25 M NaCl at pH4, while enzyme adsorbed to the same ligand and Neu2en immobilized on PAH-Sepharose remained bound even in 1 M NaCl at pH 4 making ionic interactions unlikely. This phenomenon also applies to the sialidase bound to ligands 1 and 2 (Table 1). Elution of sialidase at pH 5.3 was found for Neu-β-Me-AD- and Neu-β-Me-PAH-Sepharose and Neu2en-PAH-Sepharose at 20°C. At 4°C the sialidase eluted predominantly at pH 6.5 from Neu-β-Me-PAH-Sepharose, while with the other adsorbents it was eluted at pH 5.3 as before (Fig. 1).

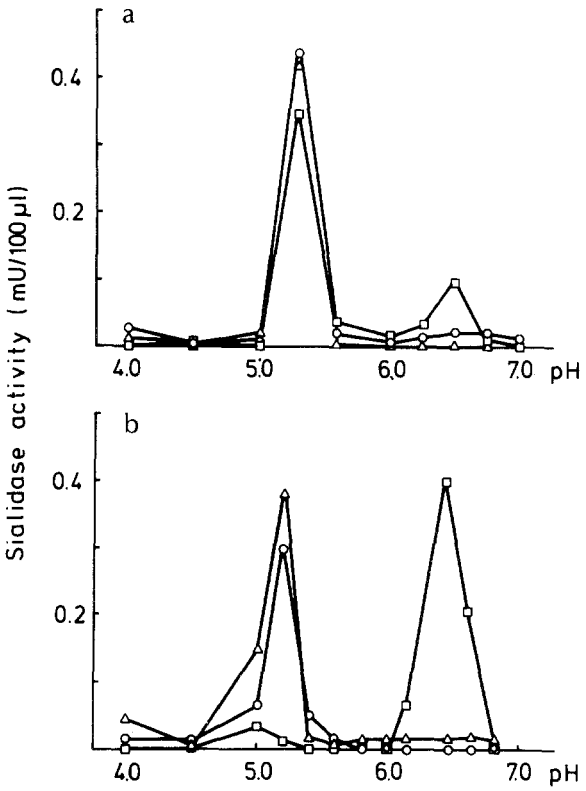

Figure 1. Binding of *C. perfringens* sialidase to immobilized sialic acid adsorbents. Elution of sialidase (25 mU) from ○, Neu-β-Me-AD-Sepharose; □, Neu-β-Me-PAH-Sepharose; and △, Neu2en-PAH-Sepharose at a) 20°C and b) 4°C. Columns (1 ml packed gel) were equilibrated in 0.05 M sodium acetate buffer, pH 4.0, at the appropriate temperature. Sialidase was applied in 5 ml of this buffer and the column washed with 10 ml of the same buffer. Elution was carried out by the same buffer at the pH values indicated using two 5 ml aliquots for each pH value.

The binding of sialidase to amino-linked sialic acid adsorbents is unexpected, as studies with synthetic substrates have shown that substitution of the amino group with large alkyl moieties reduces enzymic activity [1,39]. The binding of sialidase to adsorbents containing Neu-β-Me is interesting, as β-glycosides of sialic acid are not substrates for sialidases [1]. However, binding of *V. cholerae* sialidase and influenza virus to immobilized β-glycosides of sialic acid has also been shown [6,7,40]. The quantitative binding observed to immobilized Neu-β-Me and Neu2en ligands appears to be due to the nature of the spacer linkage to the ligand rather than the ligand or polymer alone. This is illustrated by the failure of sialyllactose (10 mM), Neu-β-Me (100 mM), Neu5Ac2en (10 mM) or Neu5Ac (100 mM) to elute bound sialidase. In addition, the hydrolysis of sialyllactose by bound sialidase further suggests that the active site is not involved in binding to the adsorbent and is available for enzymic activity. It is probable that hydrophobic interactions account for the lack of specificity of these adsorbents for *C. perfringens* sialidase alone.

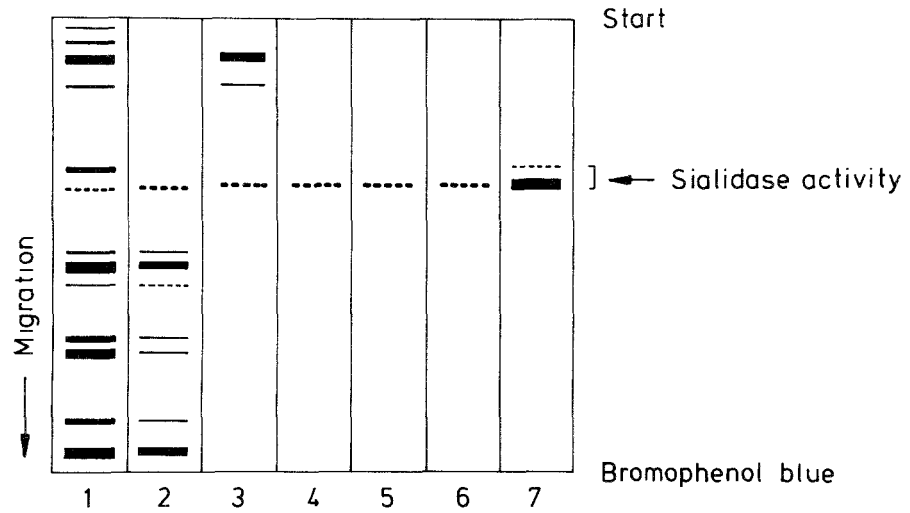

Figure 2. Electrophoretic analysis of fractions containing *C. perfringens* sialidase activity eluted from different adsorbents. A schematic representation of protein staining of gels obtained using discontinuous polyacrylamide gel electrophoresis. Separation was on 8 cm, 7.5% gels and concentration on 1 cm of 3% with 0.4 M Tris-HCl buffer, pH 8.9. The fraction was dissolved in 60 mM Tris-HCl buffer, pH 6.9, containing 0.001% bromophenol blue and 20% sucrose in a total volume of 100 µl. Concentration was at 150 V and separation at 250 V at 5°C. Gels were stained with amido black.

C. perfringens sialidase samples were: 1, starting material from Sephadex G-75 [18]; 2, Neu-β-Me-PAH-Sepharose, eluate at pH 6.5; 3, ovomucoid-Sepharose, 0.2 M NaCl eluate; 4, equine submandibular gland glycoprotein-Sepharose, 0.2 M NaCl eluate; 5, porcine submandibular gland glycoprotein-Sepharose; 0.5 M NaCl eluate; 6, bovine submandibular gland glycoprotein-Sepharose; 1 M NaCl eluate; 7, homogeneous *C. perfringens* sialidase purified from 1 by continuous electrophoresis [18]. The arrow indicates the region containing sialidase activity in sliced gels.

Due to the apparent non-affinity interaction of the adsorbents the binding of other proteins would be expected. This is reflected in the low purification factors observed (Table 1). The capacity of the PAH-Sepharose adsorbents is approximately 1 U *C. perfringens* sialidase/ml adsorbent for ligands 3 and 4 in Table 1, while the same volume of Neu-β-Me-AD-Sepharose bound 200-300 mU sialidase. The partially purified *C. perfringens* sialidase fraction used in these studies had to be diluted to 10 mU sialidase/ml buffer, pH 4.0 (45 µg protein/ml) to ensure complete binding, although higher enzyme concentrations of the purified sialidase could be quantitatively adsorbed and the same enzyme in human gas oedema serum could be adsorbed without high dilution (Schauer, Corfield and Wember, unpublished results). Using *C. perfringens* sialidase the dilution (10 mU/ml) led to a 25% loss of activity in 24 h at 4°C and large scale purification required high flow rates. Such an experiment with 8 U of *C. perfringens* sialidase on 30 ml of Neu-β-Me-PAH-Sepharose yielded 5.7 U (71%) of product with a four-fold increase in specific activity. Analysis of the product on polyacrylamide gel electrophoresis revealed several protein bands other than the sialidase (Fig. 2). Protease contamination was low (< 0.1% of the original), whereas *N*-acetyl-β-glucosaminidase levels were approximately 10% of the applied activity.

Adsorbents 2, 3 and 4 (Table 1) also bound 25 mU of purified *V. cholerae* sialidase per ml, and this enzyme was eluted under the same conditions as the *C. perfringens* sialidase.

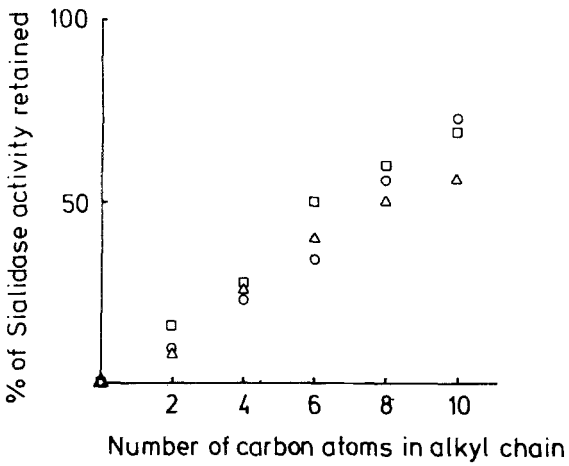

Figure 3. Binding of bacterial sialidases to alkyl agaroses. *C. perfringens* (○, 25 mU); *V. cholerae*, (□, 20 mU); and *A. ureafaciens* (△, 38 mU) sialidases were chromatographed separately on alkyl agarose columns (1 ml). Sialidases were applied in 2 ml of 0.1 M sodium acetate buffer, pH 5.0, and washed with 2 ml of the same buffer containing 0.1 M NaCl. The bound enzyme could be quantitatively released with 1 M NaCl or with 0.1 M sodium acetate buffer, pH 6.0. The results are plotted as % bound of the total activity applied, against the number of carbon atoms in the alkyl chain immobilized on agarose.

Alkyl Agaroses and 4-Nitrophenyl Oxamic Acid-AD-Sepharose

In view of the apparent hydrophobic nature of some sialidase interactions with ligands 1 to 4 (Table 1), alkyl agaroses from C_2 to C_{10} were studied. Under conditions similar to those employed for AD- and PAH-Sepharose experiments, sialidase from *C. perfringens, V. cholerae* and *A. ureafaciens* were bound with increasing efficiency by the higher alkyl agaroses (Fig. 3). Slight differences were found between the sialidases and all could be eluted using 1 M NaCl or a change of pH from 4.5 or 5.0 to 6.0. These experiments confirm that *C. perfringens* and other bacterial sialidases contain hydrophobic sites which can allow hydrophobic chromatography to be carried out.

Immobilized 4-nitrophenyl oxamic acid did not bind *C. perfringens* sialidase (Table 1) [5]. This ligand is known to inhibit some sialidases [1, 36, 42] and has been used successfully to immobilize sialidases from *V. cholerae, Trichomonas foetus, Streptococcus pneumoniae* and bovine kidney [5, 21, 43, 44] as well as viral sialidases [8, 9]. The interaction is believed to be hydrophobic, as enhanced enzymic activity of bound *V. cholerae* sialidase was observed [5] and other glycosidases are bound in the same manner [45].

Sialyl-glycoconjugate Adsorbents

Binding of *C. perfringens* sialidase to all adsorbents could be demonstrated (Table 1), although some had only low affinity and did not give quantitative adsorption of the enzyme. Optimal conditions for adsorption were found to be from pH 5.0-6.0 at 4°C. Elution could be effected by several methods: increase of pH to 8.5-9.0 in borate buffer, increasing the salt concentration in the buffer, or raising the temperature to 20-37°C. Combinations of these techniques could also be used. Increasing the salt concentration was used routinely. These results are in agreement with, and extend earlier studies using immobilized glycoconjugates [2-7,32,33].

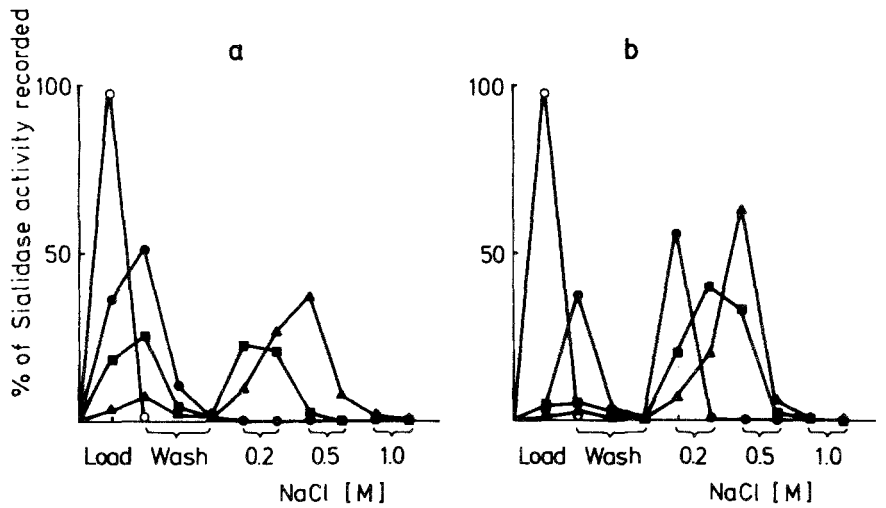

Figure 4. Effect of ligand concentration and pH on the binding of *C. perfringens* sialidase to immobilized α_1-acid glycoprotein. Sialidase (25 mU) was applied to a 1 ml packed gel column of immobilized α_1-acid glycoprotein in a) 0.05 M sodium acetate buffer, pH 6.0, or b) 0.05 M sodium acetate buffer, pH 5.0, at 4°C. The concentration of immobilized sialic acid on the adsorbents tested was: ○, zero (Sepharose 4B); ●, 0.1 μmol/ml gel; ■, 0.5 μmol/ml gel; and ▲, 1.3 μmol/ml gel. The sialidase was loaded in 2 ml of buffer (LOAD) and washed (WASH) three times with 5 ml aliquots of the same buffer. Elution was carried out using the same buffer containing 0.2, 0.5 or 1.0 M NaCl as two 5 ml aliquots for each concentration. Recovery on all columns was 80-85% of applied activity and the results are expressed as a % of the total activity recovered.

The nature of the immobilized sialyl-glycoconjugate and the concentration of immobilized sialic acid are factors which influence binding of sialidase. Plasma-type glycoproteins such as fetuin and α_1-acid glycoprotein are good substrates and are more readily hydrolysed than the mucus glycoproteins which are frequently poor substrates [1]. The binding is affected by the concentration of immobilized sialic acid (Fig. 4) and the capacity of any one adsorbent is proportional to this concentration. The susceptibility to enzyme hydrolysis during chromatography influences the capacity and efficiency of adsorption, and immobilized α_1-acid glycoprotein and fetuin, which are good substrates, are degraded during chromatography. This degradation was observed as the release of adsorbent sialic acid during elution of sialidase and a reduction in the binding capacity with repeated use of the same adsorbent batch, correlating with the amount of sialic acid lost. Mucus glycoprotein adsorbents were degraded more slowly than either of the plasma glycoproteins or the mucus glycopeptides. Correspondingly, similar concentrations of sialic acid bound as mucus glycoprotein gave complete binding of sialidase even at pH 6.0 under conditions where the α_1-acid glycoprotein adsorbent only retarded the sialidase activity. Low binding of *C. perfringens* sialidase to colominic acid-starch gel emphasises the influence of low enzyme specificity for the α(2-8)-linked sialic acid in this substrate [1], even though the concentration of immobilized sialic acid in this case is very high (Table 1, ligand 15). This is in contrast to the mucus glycoprotein adsorbents which bind sialidase efficiently but are also poor substrates relative to the plasma glycoproteins.

The degradation of fetuin and α_1-acid glycoprotein adsorbents during chromatography

has resulted in limited, if any, re-use of these adsorbents. Although quantitative binding of large amounts of sialidase can be achieved using larger volumes of adsorbent, low temperature (4°C) and rapid flow rates; degradation occurs and the resulting terminal monosaccharides (e.g. β-galactose) may bind other glycosidases (e.g. galactosidase) if re-used. This could be shown with both fetuin and α_1-acid glycoprotein adsorbents. Prolonged contact (2 h) of sialidase with the adsorbent at 20°C or 37°C led to the release of free sialic acid and sialidase. When such adsorbents were re-used the percentage of β-galactosidase bound was above 10% of that applied, compared with 0.5% and 1.0% on initial use for fetuin and α_1-acid glycoprotein, respectively (Table 1). Both fetuin and α_1-acid glycoprotein have earlier been used as affinity adsorbents for sialidase [2,4,5,32,33]. The urinary sialyl-hexasaccharide (Table 1, ligand 14) tested has a structure related to plasma type glycoproteins and was therefore rapidly desialylated with increasing amounts of sialidase and no useful chromatography resulted.

Immobilized mucus glycoprotein matrices yield the best result with sialidase. Purifications using such adsorbents (Table 1, ligands 7 to 10) were around 30-fold, which was the same as that for the enzyme purified to homogeneity by continuous polyacrylamide gel electrophoresis from the same batch of starting material (Fig. 2). Sialidase adsorbed on these matrices could be eluted using 10 mM sialyllactose, 10 mM Neu5Ac or 10 mM Neu5Ac2en indicating true affinity chromatography. The sialidase preparations obtained from these adsorbents contained no detectable protease activity, and the levels of β-galactosidase, *N*-acetyl-β-glucosaminidase and *N*-acetyl-β-galactosaminidase were below 0.1% of those applied (Table 1). This type of adsorbent has been used successfully in this laboratory in the purification of sialidases from human liver lysosomes [34] and human leucocytes [35].

Degradation of mucus glycoprotein adsorbents is low at 4°C but can be readily detected at 37°C. The mucus glycopeptide adsorbent (Table 1, ligand 9) was degraded 3-4 times more rapidly than the parent glycoprotein adsorbent (Table 1, ligand 8). This has also been noted for ovine mucus glycopeptide-Sepharose [3]. The relatively slow hydrolysis of sialic acids from mucus glycoproteins renders such affinity columns suitable for repeated use. Ovomucoid-Sepharose binds *C. perfringens* sialidase strongly, but the glycoprotein also contains substantial amounts of terminal *N*-acetylglucosamine residues [46] and is therefore a good adsorbent for *N*-acetyl-β-glucosaminidase. The resultant purification for sialidase was only six-fold. Large scale preparation of sialidase (>1 U) using ovomucoid and mucus glycoprotein adsorbents resulted in yields greater than 85% in all cases. After gel electrophoresis only one band stained for protein, corresponding to sialidase activity in the sliced gels (Fig. 2). The protein staining and enzyme activity occur at the same position as sialidase purified to homogeneity by the continuous electrophoresis method [18] using the same batch of starting material (Fig. 2).

The binding of Newcastle disease, Fowl plague and Influenza A_2 viruses to many of the sialyl-glycoconjugate matrices could be demonstrated under similar conditions to the *C. perfringens* enzyme (Table 1). The viruses could be recovered with 80% yield. No separation of sialidase from the viral envelope membrane was attempted, but this should be possible as demonstrated for Simian virus 5 HN protein on immobilized fetuin [4].

V. cholerae sialidase was also adsorbed on those matrices tested (Table 1). The enzyme was a commercially purified preparation and not a crude extract; it showed similar behaviour to the *C. perfringens* sialidase when 25 mU aliquots were chromatographed.

Other Ligands

The two immobilized plant lectins, Con A and WGA, and heparin-Sepharose showed no affinity for *C. perfringens* sialidase.

Conclusions

The immobilized ligands investigated in this study with *C. perfringens* sialidase reveal a number of characteristics of special significance for bacterial sialidases. Experiments with sialic acid and its analogues immobilized on AD- and PAH-Sepharose show that hydrophobic interactions play a significant part in the binding of sialidase. This is supported by the binding to alkyl agaroses; the presence of sialidase activity when substrate was added to the bound enzyme; and the inability of substrate, product or inhibitors to elute the enzyme from the adsorbent. The low purification and binding of other proteins to the adsorbents do not favour the use of sialic acid or analogues in general sialidase isolation. In contrast, the high purification and yield obtained on sialyl-glycoconjugate adsorbents at 4°C underlines their value on a general basis. The preferred choice of mucus glycoprotein adsorbents follows from their relatively high sialic acid content and generally slow rate of hydrolysis by sialidases [1] compared to fetuin and α_1-acid glycoprotein adsorbents. The choice of glycoprotein and the level of degradation are important factors in preventing a decrease in capacity for sialidase and the adsorption of other glycosidases. The mucus glycoprotein adsorbents tested are suitable for general use with sialidase and have already featured in the purification of two human enzymes [34,35].

Acknowledgements

The technical assistance of Marzog El Madani is gratefully acknowledged. The authors express their thanks for the gifts of viruses from Prof. R. Rott, (Giessen), and of carbohydrate compounds from Dr. J.-C. Michalski (Lille), Dr. J.-M. Beau (Orléans) and Dr. R.W. Veh (Bochum). This work was supported by the "Deutsche Forschungsgemeinschaft" (Scha 202/10-4) and the "Fonds der Chemischen Industrie".

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