RELEASE OF SACCHARIDES FROM GLYCOCONJUGATES

In many instances, it is useful to release a part or all of the oligosaccharide chains from a glycoconjugate before analyzing their structure, and the units presented in this section describe a variety of ways in which this can be achieved. Some of the methods described here (particularly the enzymatic ones) can also be applied to the study of free oligosaccharides or to oligosaccharides that are still attached to macromolecules.

Enzymes that degrade sugar chains fall into two general classes. Endoglycosidases cleave at defined sites within a sugar chain, with a specificity often based on certain features of the adjacent monosaccharide units. Thus, they are analogous to the restriction endonucleases that work on nucleic acids (*UNIT 3.1*). On the other hand, exoglycosidases specifically release single monosaccharides only when they are present as terminal units on sugar chains. Unlike the cases with exonucleases, the types of possible terminal units on oligosaccharide chains are numerous. Correspondingly, the number of available exoglycosidases with different specificities is quite large. In this section, one of the most commonly used group of exoglycosidases, the sialidases (also known as neuraminidases), is discussed in *UNIT 17.12*. Some commonly used endoglycosidases and glycoamidases for N-linked oligosaccharides (*UNIT 17.13A*) and polysaccharide lyases (*UNIT 17.13B*) are then considered.

Additional methods will be provided in future supplements (see Chapter 17 table of contents). One of the simplest and most direct ways to free an oligosaccharide chain from a glycoprotein or proteoglycan is to digest away the peptide with a broad-spectrum protease such as pronase or proteinase K (*UNIT 17.14*). In these cases, most of the peptide is removed except for the amino acids immediately surrounding the glycosylation site(s). If needed, more specific proteases (e.g., trypsin, chymotrypsin) can be used to generate glycopeptides with large stretches of amino acids still attached to the glycosylation sites. This approach is particularly useful for the detection of individual glycosylation sites on glycoproteins (*UNIT 17.14).*

There are also many classic and well-established methods for the chemical release of saccharides from glycoconjugates (*UNIT 17.15*). Some are designed to release intact sugars chains and others release the individual monosaccharide units. Although these methods tend to be somewhat less specific and potentially more destructive than the enzymatic approaches, they are cheap, convenient, and generally easy to use. These methods will be presented in future supplements.

Of course, there are many other techniques for the release of saccharides from glycoconjugates that could have been presented in this section. The current and upcoming selections were based on the criterion of broad general utility to the average molecular biologist. As methods in "glycotechnology" improve and simplify, further additions to this section may become appropriate.

Contributed by Leland D. Powell and Ajit P. Varki
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Sialidases

Sialic acids are a family of nine-carbon acidic sugars found at the nonreducing terminus of many glycoconjugates. Sialidases (a term preferred to neuraminidases) can remove these sugar units selectively from cell surfaces, membranes, or purified glycoconjugates. This may be done to analyze the sialic acids or to study the consequences of their removal. The more sensitive and specific colorimetric assays for sialic acids work on free but not on glycosidically bound molecules (*UNIT 17.16*). Although careful acid hydrolysis also can be used to release sialic acids (*UNIT 17.15*), partial destruction of modifications and/or incomplete release can be problematic. Furthermore, acid hydrolysis may alter the underlying glycoconjugate or may be incompatible with functional studies.

In this unit, sialidase digestion of purified glycoproteins is described in the basic protocol and treatment of intact cells is outlined in the alternate protocol. The physical properties of the four most useful sialidases are listed in Table 17.12.1; their relative activities against sialic acids with different modifications and in different linkages are listed in Table 17.12.2 (see also critical parameters). The choice of enzyme depends upon the nature of the sample and knowledge of the type of sialylated glycoconjugates present.

BASIC PROTOCOL

SIALIDASE TREATMENT OF PURIFIED GLYCOPROTEINS

A purified glycoprotein sample is dissolved in digestion buffer and digested with a sialidase. Controls of sample alone and enzyme alone are also prepared. Digestion of gangliosides (glycolipids with sialic acid) requires detergent (e.g., deoxycholate, cholate, or taurocholate). After digestion, the reaction is terminated and desialylation is monitored.

Materials

Sialic acid–containing sample
Sialidase digestion buffer
Sialidase (Table 17.12.1)

Additional reagents and equipment for quantitating sialic acid (*UNIT 17.16*)

1. Dissolve the sialic acid–containing sample in sialidase digestion buffer at ~0.1 to 1 mM sialic acid concentration (final).

   If the amount of sialic acid is not known, it can be estimated by acid hydrolysis (UNIT 17.15) or by quantitating the amount of sialic acid released with increasing amounts of

Table 17.12.1 General Properties of Commercially Available Sialidases

Feature	*Vibrio cholerae*	*Clostridium perfringens*	*Arthrobacter ureafaciens*	Newcastle disease virus
Form	Purified protein	Purified protein	Purified protein	Whole virion
Ca^{++} requirement	Yes	No	No	No
pH optimum	5.6[a]	4.5[b]/5.2[c]	5.0[d]	5.0–6.0
pH range	4.0–8.0	4.0–7.0	4.0–7.0	NA[e]
Maximal activity				
% at pH 5.0	>90[a]	~95[b]/>90[c]	>90[d]	NA[e]
% at pH 7.0	>70[a]	ND[e]/~40[c]	~30[d]	NA[e]

[a]In 0.1 M Tris/maleic buffer (Ada et al., 1961) and sodium acetate (Cassidy et al., 1965).
[b]In 0.1 M sodium acetate (Cassidy et al., 1965).
[c]In 0.1 M citrate-phosphate (Cassidy et al., 1965).
[d]In 0.1 M sodium acetate buffer (Uchida et al., 1979).
[e]Abbreviations: NA, not available (pH profile not published, although enzyme remains active at pH 7.0); ND, not determined.

sialidase. If the amount of sialidase added is suboptimal, free sialic acid measured (by the TBA or DMB assays, UNIT 17.16) will increase with increasing amounts of sialidase.

If the sample contains gangliosides, include 1/50 vol of 10% sodium deoxycholate (0.2% final) and place tubes in a sonicator bath for 1 to 2 min to ensure complete dispersal. If large amounts of gangliosides are present, the amount of detergent in the sample should be kept equal to or greater than the amount of ganglioside on a weight-to-weight basis. If no direct information is available concerning the amount of ganglioside present, a series of digestions with increasing amounts of detergent (e.g., 0.1%, 0.3%, 1.0%) may need to be done. Excess detergent may actually inhibit digestion. The Arthrobacter ureafaciens sialidase (see Table 17.12.1) is most active with gangliosides. Deoxycholate solutions may become cloudy in the presence of salts.

2. Add 1 to 20 mU sialidase to the sample and enzyme control tubes. Mix well.

   Select the enzyme to be used based on the properties in Table 17.12.1. The amount of enzyme to use depends on the amount of releasable sialic acid in the sample. Initially, use ~1 mU/nmol of sialic acid. Further experiments may indicate that more or less enzyme is required.

3. Prepare blank tubes containing either sample alone or enzyme alone (in sialidase digestion buffer).

   Alternative controls could include reactions incubated with inactivated sialidase (prepared by boiling 5 min) or active sialidase plus 1 mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Boehringer Mannheim), a sialidase inhibitor.

Table 17.12.2 Sialidase Action on Types of Sialic Acids[a]

Sialic acid		*Vibrio cholerae*	*Clostridium perfringens*	*Arthrobacter ureafaciens*	Newcastle disease virus
Type	Linkage(s)	Relative susceptibility			
Neu5Ac	α2-3	++++	++++	++	++++
	α2-6	+++	+++	++++	R
	α2-8	++	++	+++	+++
Neu5Gc	α2-3	++++	++++	++	++
	α2-6	+++	+++	++++	R
	α2-8	++	++	+++	++
7(9) mono-*O*-acetyl	α2-3	++	++++	++	++++
	α2-6	+	+++	++++	R
	α2-8	+	++	+++	+++
4-mono-*O*-acetyl	α2-3	R	R	R	R
	α2-6	R	R	R	R
	α2-8	R	R	R	R
7(8)9 di-*O*-acetyl	α2-3	R?	R?	++	?
	α2-6	R?	R?	++++	R
	α2-8	R?	R?	+++	?
7,8,9 tri-*O*-acetyl	α2-3	R?	R?	++?	?
	α2-6	R?	R?	++?	R
	α2-8	R?	R?	++?	?
Periodate-oxidized	α2-3	+	++	R?	?
	α2-6	+	++	R?	?
Neu5Ac-7(8)	α2-8	?	?	R?	?
O-methyl, *O*-sulfate, combinations	α2-3	?	?	?	?
	α2-6	?	?	?	?
	α2-8	?	?	?	?

[a]Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; R, practically resistant under typical digestion conditions; ?, not known. See Varki (1992) for additional details of terminology.

Table 17.12.3 Sialic Acid Standards

Glycoconjugate	Molecule	Linkages	Sialic acids[a]	Sialic acid concentration (nmol/mg)	Stock solution preparation[b]
Sialylactose (mixed isomers[c])	Oligosaccharide	α2-3 α2-6	Neu5Ac	1600	31 mg/ml in water (100×)
Fetuin[d]	Glycoprotein (N- and O-linked)	α2-3 α2-6	Neu5Ac Neu5Gc(?)	280	20 mg/ml in water (10×)
Bovine submaxillary mucin	Mucin (O-linked)	α2-6	mono- and di-*O*-acetylated Neu5Ac and Neu5Gc	150-400	20 mg/ml in water (10×)
Colominic acid	Polysaccharide	α2-8	Neu5Ac	3400	15 mg/ml in water (100×)
Mixed brain gangliosides	Glycolipid	α2-3 α2-8	Neu5Ac[f]	950[e]	5 mg/ml in 2:1:0.1 (v/v/v) chloroform/methanol/water (10×)

[a]Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid.

[b]Store all stock solutions at −20°C. Dilution to 1× will yield ~0.5 mM sialic acid.

[c]Ratio of α(2-3) to α(2-6) ~4:1, although lot-to-lot variation probably occurs.

[d]Ratio of α(2-3) to α(2-6) ~1.6:1.4 in commercial preparations, including Sigma (#F-3004) or GIBCO/BRL (Townsend et al., 1989).

[e]Depending on the manufacturer.

[f]Commercial preparations of bovine brain gangliosides have been treated with base during purification, resulting in loss of *O*-acetyl esters.

4. Incubate 3 to 4 hr at 37°C.

   Digestion of gangliosides may require 24 to 48 hr.

   For selective digestion of α(2-3) and α(2-8) linkages by the Newcastle Disease virus sialidase, use 1 to 5 mU in a 25 to 50 µl reaction and incubate 15 to 30 min at 37°C. These conditions are appropriate when the enzyme is present in excess of substrate, as is often the case when radiochemical amounts of substrate are present. The enzyme has a low level of activity against α(2-6) linkages; this becomes evident with prolonged incubations. If large amounts of sialic acids are present (e.g., >1 nmol), it will be necessary to titrate the enzyme. This can be done by setting up digests of standards containing α(2-3) or α(2-6)-linked sialic acids (Table 17.12.3) at the same concentration as the unknown sample and determining the amount of Newcastle disease virus sialidase that releases >90% of the α(2-3)-linked and <5% of the α(2-6)-linked sugar.

5. Terminate the reaction by boiling for 5 min. Check for release of sialic acid by monitoring:

   a. Sialic acid by direct measurement (*UNIT 17.16*)
   b. Shift in pI of a glycoprotein by isoelectric focusing (*UNIT 10.4*)
   c. Shift in TLC pattern for glycolipids (*UNIT 17.3*)
   d. Shift in apparent molecular weight on SDS-PAGE (*UNIT 10.2*; generally a loss of several sialic acid residues will be visible by small shifts on SDS-PAGE to either lower or higher apparent molecular weights)
   e. Alteration in lectin-binding patterns for some lectins (*UNIT 17.7*)
   f. Shift in negative charge of isolated oligosaccharides or glycolipids (*UNIT 17.17*).

SIALIDASE TREATMENT OF INTACT CELLS

ALTERNATE PROTOCOL

Although the pH optima of bacterial and viral sialidases range from 4.5 to 5.5, these enzymes can be used to treat intact, viable cells at pH 7.0. Cells are washed and resuspended in isotonic serum-free buffer, then treated with the sialidase. The reaction is terminated by centrifugation and washing.

Additional Materials

Cells, prepared as a single-cell suspension
HEPES-buffered saline (HeBS)
Culture medium appropriate for cells, with serum

1. Wash cell suspension free from serum-containing medium by centrifuging 10 min at $500 \times g$ and resuspending twice in HeBS. After the last wash, resuspend in HeBS at $0.2–1 \times 10^7$ cells/ml. Divide the cells between two tubes.

   Use HeBS containing 1 mM $CaCl_2$ if Vibrio cholerae sialidase is to be used.

   Cell number can be determined by trypan blue exclusion as described in UNIT 11.5.

2. To one tube, add sialidase. To the second tube, add no enzyme. Incubate 30 min at 37°C.

   Select the enzyme to be used based on the properties in Table 17.12.1. Initially use the sialidase in vast excess, e.g., 100 mU/ml for 10^7 cells/ml (presuming 10^7 cells will yield ~1 nmol), then titrate down for the optimum amount.

   Alternative controls could include reactions incubated with inactivated sialidase (prepared by boiling 5 min) or active sialidase plus 1 mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Boehringer Mannheim), a sialidase inhibitor.

3a. *To characterize the sialic acid molecules released from the cells:* Chill reactions to 4°C and centrifuge 10 min at $2000 \times g$, 4°C. Collect supernatant, boil 5 min to inactivate sialidase, and proceed with characterization of the released sialic acid (*UNIT 17.16*).

   It is important to inactivate the sialidase as otherwise it will continue to work on any glycoproteins shed or secreted into the medium during the 30-min incubation.

3b. *To characterize the glycoconjugates on the cell surface:* Wash cells three times in culture medium containing serum (to remove free sialidase) before proceeding with biochemical or functional assays.

REAGENTS AND SOLUTIONS

HEPES-buffered saline (HeBS)

476 mg HEPES (20 mM final)
812 mg NaCl (140 mM final)
H_2O to 100 ml
Adjust pH to 7.0 with HCl
Stable <1 year at 4°C.

Sialidase digestion buffers

***Clostridium perfringens* and *Arthrobacter ureafaciens* sialidases:** Dissolve 8.6 g sodium acetate in 100 ml water to obtain a 0.1 M sodium acetate stock solution. Adjust pH to 5.5 with 0.1 M acetic acid. Store <1 year at 4°C.

***Vibrio cholerae* sialidase**: Add 14.7 mg $CaCl_2$ dihydrate and 580 mg NaCl to 100 ml of 0.1 M sodium acetate, pH 5.5. Store <1 year at 4°C.

Newcastle disease virus sialidase: Add 2 mg of fatty acid–free BSA (Sigma A7511)

per milliliter of 0.1 M sodium acetate, pH 5.5. Any high-purity BSA can be used (contaminating glycosidases may be present in low levels in some BSA preparations). Store indefinitely at −20°C.

Sialidases

Commercial sources for sialidases include Sigma, Calbiochem, Boehringer Mannheim, and Oxford GlycoSystems (see Table 17.12.1 and *APPENDIX 4*).

***Clostridium perfringens* sialidase:** Supplied as a lyophilized powder. Reconstitute to 10 mU/µl in digestion buffer (see above) and store for several months at 4°C.

***Arthrobacter ureafaciens* sialidase:** Supplied as a lyophilized powder. Reconstitute to 1 to 10 mU/µl per manufacturer's instructions. Store for ~6 months at 4°C.

***Vibrio cholerae* sialidase:** Supplied in solution. Store per manufacturer's instructions.

Newcastle disease virus sialidase: Available commercially. Alternatively, prepare as per Paulson et al. (1982). Store stocks in 5- to 10-µl aliquots at −70°C. Thaw only once and store on wet ice at 4°C until use.

COMMENTARY

Background Information

Sialidases from many viral and bacterial strains have been described (Drzeniek, 1973). In general, bacterial enzymes have the broadest spectrum of activity, although the limited specificity of the viral enzymes can be an advantage for certain analyses.

Sialic acid is found in α(2-3) or α(2-6) linkages to neutral sugars (e.g., galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine) or in α(2-8) linkage to another sialic acid. The α(2-3)- and α(2-6)-linked sialic acid residues are found in glycoproteins (both N- and O-linked) and in glycolipids. α(2-8)-linked residues are found in glycolipids, colominic acid (from *E. coli*), and a few mammalian glycoproteins (e.g., neural cell adhesion molecule). As outlined in Table 17.12.2, not all sialidases are active towards all linkages, and these differences can be exploited in structural analyses. Sialidases are only active against α-linked sialic acids. The only natural β-linked sialic acid is in CMP (cytidine monophosphate)-sialic acid.

The common sialic acid *N*-acetylneuraminic acid (Neu5Ac) can carry modifications (primarily *O*-acetylation but also glycolylation, sulfation, and methylation; see Varki, 1992, and Table 17.12.2). Sialidases differ in their activities towards these modified sialic acids. Unfortunately, the distribution of sialic acids containing these modifications has not been well-characterized in most mammalian systems.

Critical Parameters

Several caveats must be kept in mind in using sialidases.

Cleavage. The relative catalytic rates of the different enzymes for the various linkage types—i.e., α(2-3), α(2-6), and α(2-8)—are not of much consequence in most instances, because the enzymes are usually used in excess. Important exceptions to this include: (1) The Newcastle disease virus sialidase preferentially cleaves α(2-3) and α(2-8) linkages. However, α(2-6)-linked sialic acid will be hydrolyzed slowly if an excessive amount of enzyme is employed or if incubations are continued >30 min. (2) The *Vibrio cholerae* enzyme does not cleave the α(2-3)-linked "internal" sialic acid of extended gangliosides (e.g., G_{M1}), although it is active against the oligosaccharide if ceramide is removed. (3) The α(2-8) linkages of colominic acid and b series gangliosides are relatively resistant to release. Combined treatments with sialidases and endosialidases (Troy, 1992) may be used for polysialic acids with more than five sialic acid units. (4) Ganglioside-bound sialic acids may require detergents for complete release. Deoxycholate and cholate work the best.

Effect of substitution. Substitutions have variable effects upon release depending on the enzyme used, as summarized in Table 17.12.2. The decrease in rate with the *N*-glycolyl modification is not relevant in using any of the enzymes. The decrease in rates for the 9- or 7-mono-*O*-acetyl substitutions are also not practically relevant, except for the *Vibrio* enzyme, and particularly with gangliosides. On the other hand, the 4-mono-*O*-acetyl substitution causes complete resistance to all known sialidases. The effects of di- and tri-*O*-acetyl

substitutions have not yet been carefully studied, and it is not safe to extrapolate from data on mono-*O*-acetyl molecules. Likewise, combinations of substitutions (e.g., *N*-glycolyl and *O*-acetyl) have not been studied carefully. Finally, there is no information on the rarer types of sialic acids (e.g., *O*-methyl and *O*-sulfate substituted). If *O*-acetylation is suspected, prior treatment of the glycoconjugates with base (*UNIT 17.15*) will cause de-*O*-acetylation and improve release.

Controls. Whenever possible, controls such as the sample without enzyme and the enzyme alone must be studied in parallel to be sure that the sialic acids detected are actually released from the sample. This is particularly important when ultrasensitive methods (such as TBA with HPLC detection or DMB derivatization; *UNIT 17.16*) are employed. These methods are so sensitive that "environmental contamination" with sialic acids can become a problem.

Analysis of results. It is useful to compare the amount of sialic acid released (*UNIT 17.16*) by sialidase with the amount released by mild acid hydrolysis (*UNIT 17.15*). If the amount released by the sialidases is significantly less than that released by mild acid, then the sialic acid residues may be resistant due to (1) incorrect choice of sialidase, (2) modification of the sialic acid residues, (3) steric hindrance, or (4) suboptimal conditions for digestion.

It may be important to demonstrate that the observed effect produced by sialidase treatment (e.g., shift in isoelectric point on a focusing gel or a functional response) is in fact due to the sialidase activity. Treatment with heat-inactivated enzyme (prepared by boiling for 5 min) or inhibition with 1 mM 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, a specific inhibitor of sialidases, can be used. The reaction should be titrated so that just enough sialidase is being used for the observed result, and then the effect of the inhibitor assessed.

Sialidase inhibitors. Inhibitors of sialidases do exist. Dextran sulfate inhibits the *Vibrio cholerae* sialidase. Other polyanions (e.g., DNA, RNA, and proteoglycans) may also inhibit this or other sialidases. Free sialic acid also can inhibit the *Vibrio cholerae* enzyme, with a K_i of ~5 mM.

Agglutination. Treating intact cells with Newcastle disease virus can cause agglutination of cells. This effect should not be confused with clumping due to cell death.

Buffer systems. The sialidases are compatible with several different buffer systems, with some differences in pH optima noted (Table 17.12.1). *Vibrio cholerae* sialidase works well in sodium acetate and Tris/maleate at pH 5.0 to 6.0 but is considerably more active in Tris/maleate at pH >5.0. *Clostridium perfringens* sialidase has pH optima of 4.5 in sodium acetate but 5.5 in citrate phosphate. *Arthrobacter ureafaciens* sialidase digestion proceeds maximally at pH 5.5, yet retains >50% of activity at pH 4.5 and 7.0. For all sialidases, alternative choices include sodium acetate, ammonium acetate, Tris, HEPES, phosphate, and cacodylate. Acetate interferes with HPLC on AX-5 resin (*UNIT 17.12*) and β-hexosaminidase digestion (*UNIT 17.13*).

Troubleshooting

Failure to digest a known sialoglycoconjugate to completion could be due to failure to add sufficient sialidase, use of an old or improperly reconstituted and/or stored enzyme aliquot, or inactivation of the enzyme after addition. If the sample is an unknown but potentially contains >1 nmol of releasable sialic acid, a titration comparing increasing amounts of sialidase versus sialic acid released is useful. Sialidases can lose activity rapidly (e.g., <1 hr) after dilution into an assay mix; inclusion of BSA will help prevent this inactivation. Some manufactures recommend that the *Arthrobacter ureafaciens* and *Clostridium perfringens* sialidases be reconstituted with BSA for stability on storage.

Anticipated Results

If the type of sialidase used is tailored to the type of sialic acid and linkage, it should be possible to obtain nearly complete release of these residues.

Time Considerations

Once the appropriate buffers and standards are made, the actual digestions will take 30 min to 4 hr or longer (especially for gangliosides). Time required for analysis of the digested product(s) will depend on the specific analytical method employed (as listed in step 6 of the basic protocol).

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