Selectin Biosciences Inc.

Enzymatic Deglycosylation Kit

Contains all enzymes needed to completely remove all N- & simple O-linked carbohydrates from glycoproteins

- Deglycosylates ≥ 2 mg Glycoprotein
- Single reaction at neutral pH
- Native & denaturing protocols
- No degradation of protein
- Removes O-linked sugars containing polysialic acid
- Control glycoprotein provided

Product Code: GE 52

Kit Contents

PNGase F (5,000 U/ml)	20 µL
Endo-O-Glycosidase (1.25 U/ml)	20 µL
Alpha(2-3, 6, 8, 9) Neuraminidase (5 U/ml)	20 µL
Beta(1-4) Galactosidase (3 U/ml)	20 µL
Beta-N-Acetylglucosaminidase (45 U/mL)	20 µL
Bovine Fetuin Control (10 mg/mL)	50 µL
5X Reaction Buffer 7 (0.25 M Sodium Phosphate pH 7)	0.2 mL
Denaturation Solution (2% Sodium Dodecylsulfate [SDS] and 1 M β-mercaptoethanol)	0.1 mL
Triton X-100 (15% Solution)	0.1 mL

Storage Conditions

Kits are shipped with a cold pack for next day delivery and should be stored at 4°C. Do not freeze.

They may be used for at least 1 year when stored in this manner. Sterile pipette tips should be used as no preservatives have been added.

Introduction

Carbohydrates in the form of asparagine-linked (N-linked) or serine/threonine-linked (O-linked) oligosaccharides are major structural components of many eukaryotic proteins. They perform critical biological functions in protein sorting, immune recognition, receptor binding, inflammation, pathogenicity and many other processes. The diversity of oligosaccharide structures often results in heterogeneity in the mass and charge of glycoproteins. N-linked oligosaccharides may contribute 3.5 kD or more per structure to the mass of a glycoprotein (see Figure 1). Variations in the structures and different degrees of saturation of available glycosylation sites in a glycoprotein all contribute to mass heterogeneity. The presence of sialic acid (N-acetylneuraminic acid) affects both the mass and charge of a glycoprotein. Other modifications to the carbohydrate such as phosphorylation or sulfation also affect charge.

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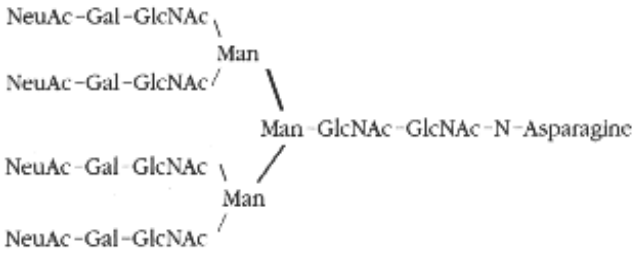
Figure 1 Tetraantennary N-linked Sugar

Gal - Galactose; Man - Mannose; GalNAc - N-acetylgalactosamine; GlcNAc - N-acetylglucosamine; NeuAc - N-acetylneuraminic Acid (Sialic Acid)



O-linked sugars, although usually less massive than N-linked structures, may be more numerous and are also heterogenous in structure (see Figures 2 & 3).

Figure 2 Di- and Trisialylated O-linked Core (core shown in bold)

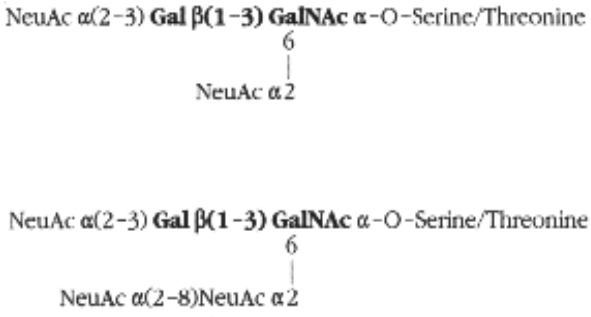
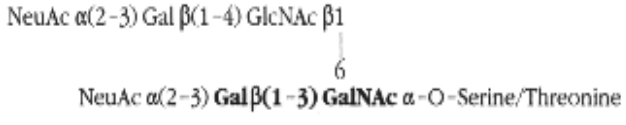


Figure 3 O-linked Hexasaccharide



To study the structure and function of a glycoprotein, it is often desirable to remove all or a select class of oligosaccharides. This approach allows the assignment of specific biological functions to particular components of the glycoprotein. For example, the loss of ligand binding to a glycoprotein after removal of sialic acid may implicate this sugar in the binding process.

- Researchers may wish to remove sugars from glycoproteins for a number of reasons:
- For simplifying amino acid sequence determination of glycoproteins
- To remove heterogeneity in glycoproteins for X-Ray crystallographic analysis
- To remove carbohydrate epitopes from antigens
- To enhance or reduce blood clearance rates of glycoprotein therapeutics[15].
- To investigate the role of carbohydrates in enzyme activity and solubility
- To investigate ligand binding
- For quality control of glycoprotein pharmaceuticals
- To study the peptide portion of the glycoprotein by SDS PAGE

Deglycosylation Methods

Both chemical and enzymatic methods exist for removing oligosaccharides from glycoproteins. Hydrazinolysis of glycoproteins[9], although capable of removing both N- and O-linked sugars, it results in the complete destruction of

the protein component and is therefore not suitable if recovery of the protein is desirable. Milder chemical methods such as trifluoromethanesulphonic acid (TFMS)[12], even when optimized, result in incomplete sugar removal and partial protein destruction. The amino acid-linked sugar residue of both N- and O-linked oligosaccharides is retained. Only the enzymatic method provides complete sugar removal with no protein degradation.

Enzymatic Removal of N-linked Oligosaccharides

Use of the enzyme PNGase F is the most effective method of removing virtually all N-linked oligosaccharides from glycoproteins[17]. The oligosaccharide is left intact and therefore suitable for further analysis (the asparagine residue from which the sugar was removed is deaminated to aspartic acid, the only modification to the protein). A tripeptide with the oligosaccharide-linked asparagine as the central residue is the minimal substrate for PNGase F. However, oligosaccharides containing a fucose α(1-3)-linked to the asparagine-linked N-acetylglucosamine, commonly found in glycoproteins from plants or parasitic worms, are resistant to PNGase F[1]. Endoglycosidase A, isolated from almond meal, must be used in this situation[16]. This enzyme, however, is ineffective when sialic acid is present on the N-linked oligosaccharide.

Steric hindrance slows or inhibits the action of PNGase F on certain residues of glycoproteins. Denaturation of the glycoprotein by heating with SDS and βME greatly increases the rate of deglycosylation. For some glycoproteins, no cleavage by PNGase F occurs unless the protein is denatured. For others, some or all of the oligosaccharides can be removed from the native protein after extensive incubations of three days or longer. PNGase F will remain active under reaction conditions for at least three days allowing extended incubations of native glycoproteins. In general, it appears that particular residues, due to their location in the native protein structure, are resistant to PNGase F and cannot be removed unless the protein is denatured (see Figure 4, lane 6).

NOTE: A non-ionic detergent such as Triton X-100 must be added in excess to the SDS-denatured glycoprotein prior to the addition of PNGase F to complex any free SDS. Reduction in the rate of PNGase F cleavage will result if this procedure is not followed.

Other commonly used endoglycosidases such as Endoglycosidase H[8] and the Endoglycosidase F[18] series are not suitable for general deglycosylation of N-linked sugars because of their limited specificities and because they leave one N-acetylglucosamine residue attached to the asparagine.

O-linked Oligosaccharides

There is no enzyme comparable to PNGase F for removing intact O-linked sugars. Monosaccharides must be removed by a series of exoglycosidases until only the Galβ(1-3)GalNAc core remains attached to the serine or threonine. Endo-O-Glycosidase (endo-α-N-acetylgalactosaminidase)[6] can then remove the core structure intact with no modification of the serine or threonine residues. Denaturation of the glycoprotein does not appear to significantly enhance de-O-glycosylation.

Any modification of the core structure will block the action of Endo-O-Glycosidase. By far the most common modification of the core Galβ(1-3)GalNAc is a mono-, di- or trisialylation[3,10,11,13].

These residues are easily removed by a suitable sialidase. The trisialyl structure can only be

removed by the Arthrobacter ureafaciens sialidase[19] since only this enzyme is capable of efficient cleavage of the NeuAcα(2-8)NeuAc bond.

A less common but widely distributed O-linked hexasaccharide structure contains β(1-4)-linked galactose and β(1-6)-linked N-acetylglucosamine as well as sialic acid[5,20].

Complete removal of this O-linked structure or its derivatives would require, in addition to sialidase, a β(1-4)-specific galactosidase and an N-acetylglucosaminidase. The galactosidase must be β(1-4)-specific since a non-specific galactosidase would remove the β(1-3)galactose from the core Galβ(1-3)GalNAc leaving O-linked GalNAc, which cannot be removed by Endo-O-Glycosidase. This kit provides the appropriate additional enzymes and reagents for degrading these and any other O-linked structures containing β (1-4)-linked galactose and β -linked N-acetylglucosamine such as polylactosamine.

Other rare modifications that have been found on O-linked oligosaccharides include α-linked galactose and α-linked fucose[4,20]. Directly O-linked N-acetylglucosamine (found on nuclear proteins)[7] and α-linked N-acetylgalactosamine (found in mucins) have also been reported. Addition of the appropriate enzymes (not included in the kit) would be necessary for complete de-O-glycosylation if these residues are present. Fucose[14] and mannose[2] directly O-linked to proteins cannot presently be removed enzymatically.

Monitoring Deglycosylation

The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS polyacrylamide gels (PAGE). The amount of enzyme added to a reaction in the kit is <200 ng for each enzyme; bands corresponding to the enzymes in the gels should be barely visible compared with the glycoprotein.

As shown in Figure 4 for Bovine Fetuin, sequential addition of each of the three enzymes PNGase F, α (2-3,6,8,9) neuraminidase and Endo-O-Glycosidase results in a noticeable increase in mobility. The greatest shift (lane 3) is a result of removal of N-linked sugars by PNGase F. Removal of sialic acid from O-linked sugars by α (2-3,6,8,9) results in the shift shown in lane 4.

Finally, removal of the O-linked core Galβ(1-3)GalNAc by Endo-O-Glycosidase is responsible for the small shift in lane 5. The ability to detect obvious mobility shifts when the disaccharide core structure is removed will depend on the size of the protein and the relative mass contribution of the disaccharides removed. Thus, it may be difficult to establish the presence of O-linked sugars based solely on mobility shifts following Endo-O-Glycosidase treatment of very large proteins with small numbers of O-linked sugars.

Other methods can be used in conjunction with SDS PAGE gels to monitor deglycosylation. These methods, used in gel or blot formats, directly detect the carbohydrate portion of the glycoprotein. In each method, the carbohydrate is oxidized with periodate. The oxidized carbohydrate is either directly stained (Alcian Blue or silver stain) or is reacted with biotin hydrazide, which biotinylates the sugar. An enzyme-linked streptavidin conjugate and the appropriate indicator substrate is used to detect the carbohydrate in a blot. Completely deglycosylated protein produces no signal with these methods. Kits are available from several manufacturers for performing this type of analysis.

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Figure 4 Enzymatic Deglycosylation of Bovine Fetuin

Lane 1 - Molecular Weight Markers

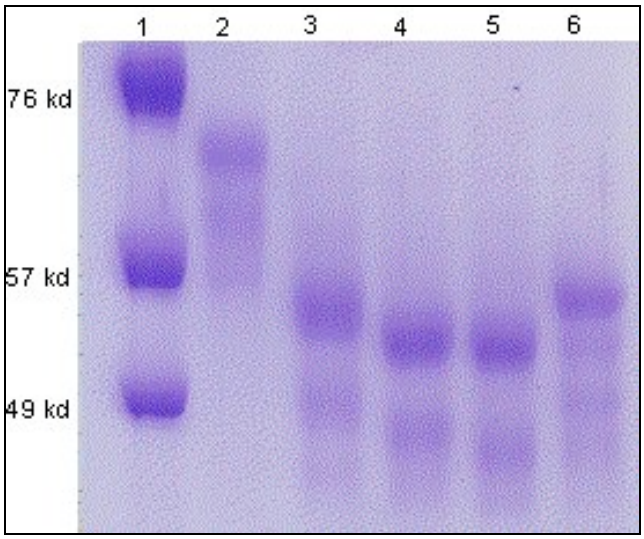
Lane 2 - Denatured Bovine Fetuin (DBF)

Lane 3 - DBF + PNGase F (3 hour incubation)

Lane 4 - DBF + PNGase F + Alpha(2-3, 6, 8, 9) neuraminidase (3 hour)

Lane 5 - DBF + PNGase F + Alpha(2-3, 6, 8, 9) neuraminidase + Endo-O-Glycosidase (3 hour)

Lane 6 - Native Fetuin + PNGase F + Alpha(2-3, 6, 8, 9) neuraminidase + Endo-O-Glycosidase (3 day incubation)



Protocols

Denaturing Protocol

1. Dissolve 100 µg or less of a glycoprotein in 30 µL deionized water in an Eppendorf tube.
2. Add 10 µL 5X Reaction Buffer 7 and 2.5 µL Denaturation Solution. Mix gently.
3. Heat at 100°C for 5 minutes.

NOTE: Some proteins may precipitate when heated with SDS. In this event, omit the heat treatment and increase the incubation time to 24 hours after adding enzymes.

4. Cool to room temperature. Add 2.5 µl Triton X-100 solution. Mix gently.

NOTE: Failure to add Triton X-100 may result in the reduction of activity of some enzymes.

5. Add 1 µL each of each enzyme.
6. Incubate for 3 hours at 37°C.
7. Analyze by method of choice.

Alternatively, the enzymes may be added individually or sequentially in order to determine what types of oligosaccharides are present on the glycoprotein as in Figure 4.

Non-denaturing Protocol

1. Dissolve 100 µg or less of a glycoprotein in 35 µL deionized water in an Eppendorf tube.
2. Add 10 µL 5X Reaction Buffer 7.
3. Add 1 µL of each enzyme.
4. Incubate for 1-5 days at 37°C.

An aliquot should be deglycosylated using the denaturing protocol to provide a gel standard for the fully deglycosylated protein. The position of the native protein can then be compared with this

standard to judge the extent of deglycosylation (see Figure 4, lane 6).

Kit Capacity

The quantity of enzymes recommended in the protocols is sufficient to deglycosylate approximately 100 µg of an average glycoprotein in the time given. PNGase F cleavage is generally the rate limiting reaction due to the slow removal of some sterically hindered N-linked residues, even when the glycoprotein is denatured. Since all of the enzymes retain activity under reaction conditions for several days, a much larger quantity of glycoprotein may be deglycosylated if incubation is extended. Conversely, there is no need to use the recommended amounts of enzymes if quantities much less than 100 µg of glycoprotein are being cleaved. The enzymes can be diluted into 1X Reaction Buffer 7. They will remain stable in diluted form at 4°C.

Bovine Fetuin Control Protein

Bovine Fetuin contains sialylated N- and O-linked oligosaccharides[13].

NOTE: Commercial preparations of Fetuin contain proteases which will eventually degrade the protein. The Fetuin Control has been heat treated at 90°C for 10 minutes to inactivate the proteases. The Fetuin solution can be stored at 4°C.

Enzyme Specificities

PNGase F *Elizabethkingia miricola*)[17] cleaves all asparagine-linked complex, hybrid or high mannose oligosaccharides unless α(1-3) core fucosylated[1]. Asparagine must be peptide bonded at both termini. MW = 34 kDa.

Alpha (2-3, 6, 8, 9) Neuraminidase (recombinant from *Arthrobacter ureafaciens*)[19] cleaves all non-reducing terminal branched and unbranched sialic acids. MW = ~70 kDa.

Endo-O-Glycosidase (recombinant from *Streptococcus pneumonia*)[4,6] cleaves serine- or threonine-linked unsubstituted Galβ(1-3)GalNAcα. MW = ~180 kDa

β(1-4)-Galactosidase (recombinant from *Streptococcus pneumonia*)[4] releases only β(1-4)-linked, non-reducing terminal galactose. MW = ~240 kDa

Glucosaminidase (recombinant from *Streptococcus pneumonia*)[4] cleaves all non-reducing terminal β-linked N-acetylglucosamine residues. MW = ~140 kDa

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This product is intended for in vitro research only.

Revision 5/22/12