

Product Information

Enzymatic Protein Deglycosylation Kit

Catalog Number **EDEGLY**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

The EDEGLY kit contains all the enzymes and reagents needed to completely remove all N-linked and simple O-linked carbohydrates from glycoproteins, as well as cleavage of complex Core 2 O-linked carbohydrates including those containing poly lactosamine. This kit has the following features:

- Deglycosylates two or more mg of glycoprotein
- Single reaction at neutral pH
- Native & denaturing procedures
- No degradation of protein
- Removes O-linked sugars containing polysialic acid
- Control glycoprotein provided

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

To study the structure and function of a glycoprotein, it is often desirable to remove all or just 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.

Removal of carbohydrates from glycoproteins is useful 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 therapeutics¹
- 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

Both chemical and enzymatic methods exist for removing oligosaccharides from glycoproteins. Hydrazinolysis of glycoproteins,² although capable of removing both N-linked and O-linked sugars, 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 trifluoromethanesulfonic acid (TFMS),³ even when optimized, result in incomplete sugar removal and partial protein destruction. The amino acid-linked sugar residue of both N-linked and O-linked oligosaccharides is retained. Only the enzymatic method provides complete sugar removal with no protein degradation.

Use of the enzyme PNGase F is the most effective method of removing most N-linked oligosaccharides from glycoproteins.⁴ 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.

Oligosaccharides containing a fucose $\alpha(1\rightarrow3)$ -linked to the asparagine-linked N-acetylglucosamine, commonly found in glycoproteins from plants or parasitic worms, are resistant to PNGase F. Endoglycosidase A, isolated from almond meal, must be used in this situation.⁵ 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 2-mercaptoethanol 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 incubation 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 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).

Other commonly used endoglycosidases such as Endoglycosidase H and the Endoglycosidase F 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.^{6,7}

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 Gal- $\beta(1\rightarrow3)$ -GalNAc core remains attached to the serine or threonine. O-Glycosidase (endo- α -N-acetylgalactosaminidase)⁸ 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 O-deglycosylation. Any modification of the core structure will block the action of O-Glycosidase. By far the most common modification of the core Gal- $\beta(1\rightarrow3)$ -GalNAc is a mono, di, or trisialylation.⁹⁻¹² These residues are easily removed by a suitable neuraminidase. The trisialyl structure can only be removed by the neuraminidase from *Arthrobacter ureafaciens* (Catalog Number N8271, α -(2 \rightarrow 3,6,8,9)-Neuraminidase or Sialidase A),¹³ since only this enzyme is capable of efficient cleavage of the NeuAc- $\alpha(2\rightarrow8)$ -NeuAc bond.

A less common, but widely distributed O-linked hexasaccharide structure contains $\beta(1\rightarrow4)$ -linked galactose and $\beta(1\rightarrow6)$ -linked N-acetylglucosamine as well as sialic acid.^{14,15} Complete removal of this O-linked structure or its derivatives would require, in addition to neuraminidase, a $\beta(1\rightarrow4)$ -specific galactosidase and an N-acetylglucosaminidase. The galactosidase must be $\beta(1\rightarrow4)$ -specific since a non-specific galactosidase would remove the $\beta(1\rightarrow3)$ -galactose from the core Gal- $\beta(1\rightarrow3)$ -GalNAc leaving O-linked GalNAc, which can not be removed by O-Glycosidase. $\beta(1\rightarrow4)$ -Galactosidase (Catalog Number G0413) and β -N-Acetylglucosaminidase (Catalog Number A6805) are used for the degradation of these and any other O-linked structures containing $\beta(1\rightarrow4)$ -linked galactose or 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.^{15,16} Directly O-linked N-acetylglucosamine (found on nuclear proteins)¹⁷ 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 O-deglycosylation if these residues are present. Fucose¹⁸ and mannose¹⁹ directly O-linked to proteins can not presently be removed enzymatically.

The enzymes contained in this kit have the following properties:

PNGase F (*Elizabethkingia miricola*, *Elizabethkingia miricola* was formerly known as *Elizabethkingia*, *Chryseobacterium*, or *Flavobacterium meningosepticum*)⁴ cleaves all asparagine-linked complex, hybrid, or high mannose oligosaccharides unless the core GalNAc is fucosylated.¹ The asparagine must be peptide bonded at both termini. The molecular mass of PNGase F is ~36 kDa.

α -(2 \rightarrow 3,6,8,9)-Neuraminidase (recombinant from *Arthrobacter ureafaciens*) cleaves all non-reducing terminal branched and unbranched sialic acids. The molecular mass is ~60 kDa and 69 kDa (2 active forms).

O-Glycosidase (recombinant from *Streptococcus pneumoniae*)^{8,16} cleaves serine or threonine-linked unsubstituted Gal- $\beta(1\rightarrow3)$ -GalNAc- α -. The molecular mass is ~180 kDa.

$\beta(1\rightarrow4)$ -Galactosidase (recombinant from *Streptococcus pneumoniae*)¹⁶ releases only $\beta(1\rightarrow4)$ -linked, non-reducing terminal galactose. The molecular mass is ~350 kDa.

β -N-Acetylglucosaminidase (recombinant from *Streptococcus pneumoniae*)¹⁶ cleaves all non-reducing terminal β -linked N-acetylglucosamine residues. The molecular mass is ~140 kDa.

Figure 1.

Tetraantennary N-linked Sugar

Gal - Galactose

Man - Mannose

GalNAc - N-acetylgalactosamine

GlcNAc - N-acetylglucosamine

NeuAc - N-acetylneuraminic Acid (Sialic Acid)

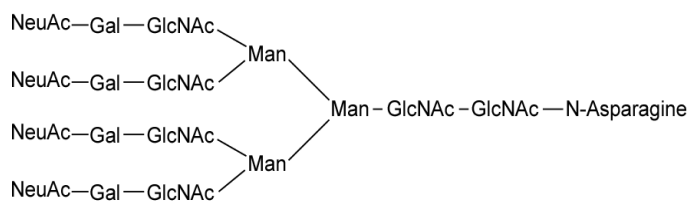


Figure 2.

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

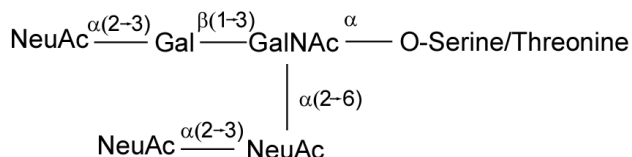
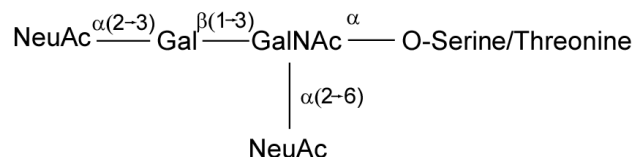
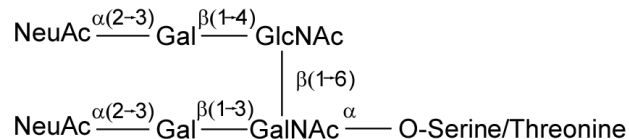


Figure 3.

O-linked Core-2 Hexasaccharide



Components

PNGase F (20 μ l) (Catalog Number P2619)	1 vial
O-Glycosidase (Endo-O-Glycosidase) (Catalog Number G1163)	20 μ l
α -(2 \rightarrow 3,6,8,9)-Neuraminidase (Sialidase A) (Catalog Number N8271)	20 μ l
Fetuin Control, 10 mg/ml solution (Catalog Number F4301)	0.5 mg
5 \times Reaction Buffer (Catalog Number R2651)	0.2 ml
Denaturation Solution (Catalog Number D6439)	0.1 ml
TRITON [®] X-100 (15% Solution) (Catalog Number T3319)	0.1 ml
β -(1 \rightarrow 4)-Galactosidase (Catalog Number G0413)	20 μ l
β -N-Acetylglucosaminidase (Catalog Number A6805)	20 μ l

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The EDEGLY kit ships on wet ice and storage at 2–8 °C is recommended. This kit may be used for at least 1 year when stored as indicated.

Procedures

The quantity of enzymes recommended in these procedures is sufficient to deglycosylate ~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 time 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 1× Reaction Buffer. They will remain active in diluted form at 2–8 °C.

Bovine fetuin is supplied as a control glycoprotein. It contains sialylated N-linked and O-linked oligosaccharides.¹² It is supplied as a 10 mg/ml solution in water. 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 these proteases. The fetuin solution can be stored at 2–8 °C after reconstitution.

Deglycosylation under Denaturing Conditions

1. Dissolve 100 µg or less of a glycoprotein in 30 µl of deionized water in an Eppendorf® tube.
2. Add 10 µl of 5× Reaction Buffer and 2.5 µl of 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 step and increase the incubation time to 24 hours after adding the enzymes.
4. Cool to room temperature. Add 2.5 µl of the TRITON X-100 solution. Mix gently.
Note: Failure to add the TRITON X-100 solution may result in the reduction of activity of some enzymes.
5. Add 1 µl each of the PNGase F, O-Glycosidase, and α -(2→3,6,8,9)-Neuraminidase solutions. If complex Core 2 O-linked carbohydrates are to be hydrolyzed, add 1 µl each of β -N-Acetylglucosaminidase and β -(1→4)-Galactosidase.
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 seen in Figure 4.

Deglycosylation under Native Conditions

1. Dissolve 100 µg or less of a glycoprotein in 35 µl of deionized water in an Eppendorf tube.
2. Add 10 µl of 5× Reaction Buffer.
3. Add 1 µl each of PNGase F, O-Glycosidase, and α -(2→3,6,8,9)-Neuraminidase solutions. If complex Core 2 O-linked carbohydrates are to be hydrolyzed, add 1 µl each of β -N-Acetylglucosaminidase and β -(1→4)-Galactosidase.
4. Incubate for 1–5 days at 37 °C.

An aliquot should be deglycosylated using the denaturing procedure 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).

Results

The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels. The amount of enzyme added to a reaction in the EDEGLY kit is <200 ng for each enzyme and the bands corresponding to these 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 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)-Neuraminidase results in the shift in lane 4.

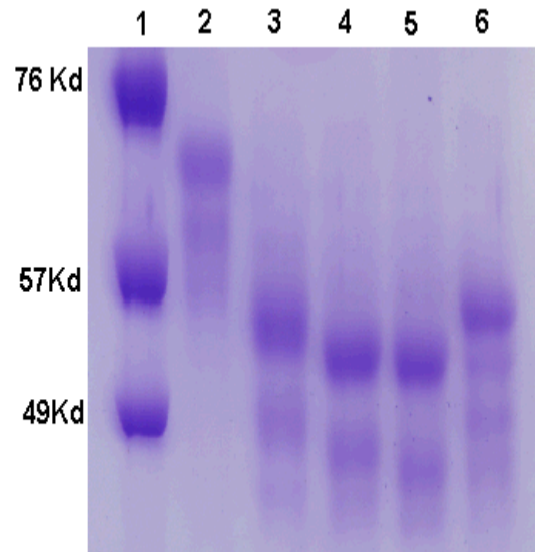
Finally, removal of the O-linked core Gal- β (1→3)-GalNAc by O-Glycosidase is responsible for the small shift in lane 5. The ability to detect obvious mobility shifts when the disaccharide core structure is removed will depend on the size of the protein and the relative mass contribution of the disaccharides removed. Thus, it may be difficult to establish the presence of O-linked sugars based solely on mobility shifts following 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 (Catalog Number B7639), which biotinylates the sugar. An enzyme-linked streptavidin conjugate and the appropriate indicator substrate are used to detect the carbohydrate in a blot. Completely deglycosylated protein produces no signal with these methods.

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 + α -(2→3,6,8,9)-Neuraminidase (3 hour incubation)
- Lane 5 - DBF + PNGase F + O-Glycosidase + α -(2→3,6,8,9)-Neuraminidase (3 hour incubation)
- Lane 6 - native fetuin + PNGase F + O-Glycosidase + α -(2→3,6,8,9)-Neuraminidase (3 day incubation)



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