

Chemical and Enzymatic Release of Glycans from Glycoproteins

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1. Introduction

The majority of proteins are posttranslationally modified, and the most significant modification to many secreted and membrane-associated proteins of eukaryotic cells is glycosylation, that is, the attachment of one or more oligosaccharide (glycan) chains. Glycans may be attached to the peptide backbone through different types of linkage but they usually are subdivided into those attached to glycoproteins primarily through an amide linkage to asparagine residues (*N*-linked glycans), and those attached through an *O*-glycosidic linkage to serine or threonine residues (*O*-linked glycans) or where the carbohydrates form part of a glycosylphosphatidyl inositol moiety (GPI) attached to the C-terminus of the peptide. Other types of linkage occur in certain other glycoconjugates such as the linkage to hydroxylysine residues in collagen and β -xylose of glycosaminoglycan chains in proteoglycans to serine residues in the peptide core.

The structural diversity of glycans attached to proteins (**1**), as well as the fact that each glycosylated polypeptide is generally associated with a population of different glycan structures (**2**) leads to the considerable glycosylation heterogeneity observed in many glycoproteins. With current techniques the analysis is generally not possible on the intact glycoprotein. For this reason oligosaccharide analysis is performed mainly following release of the oligosaccharides from the polypeptide. A number of important considerations need to be taken into account regarding the release procedure, and the following criteria may be set:

1. Release should be nonselective with regard to the types of glycan; otherwise a representative profile will not be obtained.
2. The release should cause no modification of the glycan.
3. It should be suitably efficient to allow recovery of sufficient material for study of the chosen sample.
4. The peptide material should be separated from the released glycans.

An additional consideration is that a free reducing terminal will make subsequent derivatization for analysis of the glycans much simpler and is very desirable.

Techniques for glycan release have been devised based on either an enzymatic or a chemical procedure. Each type of technique has its own merits, and the choice of technique will depend on such factors as the type of glycosylation present and the nature and amount of the sample. In this chapter we concentrate on the release of the *O*- and *N*-linked and GPI-linked glycans attached to glycoproteins.

Historically, chemical methods have been used to release *O*- and *N*-linked oligosaccharides. A number of chemical techniques for release have been described and used for several years but principally those most commonly used are hydrazinolysis and alkali/reducing conditions (β -elimination) (3,4). The use of anhydrous hydrazine for release of *N*-linked glycans was developed mainly by the group of Kobata (4) and has now been applied to the analysis of a large number of glycoproteins by many groups. It is thus a well established and validated technique. More recently it has been shown (5,6) that the technique may be modified for the release of *O*-glycan structures.

In the last two decades, a growing repertoire of enzymes, including endoglycosidases and glycosamidases, able to release glycoprotein oligosaccharides under mild conditions have been available. The use of these enzymes enables convenient and nonselective release of *N*-linked oligosaccharides from glycoproteins. Some of these have a high degree of specificity with respect to the type of *N*-linked oligosaccharides released. These have been well characterized and some of them have been cloned (3). The specificity may cause problems; for example, endoglycosidases able to release *O*-linked sugars exhibit very restricted substrate specificity that limits their use.

In the cases when the protein is difficult to purify or when there are limited amounts of sample, the *N*-glycan may be released directly from a band on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel or a spot on two-dimensional electrophoresis using peptide *N*-glycosidase F (PNGase F) (7). Following release, sequential exoglycosidase digestion using highly specific enzymes can be used for simultaneously sequencing the glycan in a standard panel of enzyme arrays, with analysis of the product using high-performance liquid chromatography (HPLC). The new approaches involve the digestion of aliquots of a total pool of oligosaccharides (fluorescently labeled)

with a series of multiple enzyme arrays (8,9). New techniques for the oligosaccharide chain-cleaving enzymes are constantly developed using bacterial cells as a source.

The first described endo- β -*N*-acetylglucosaminidase able to release *N*-linked glycans from glycoproteins was endo D, isolated from the genus *Diplococcus*. The specificity of this enzyme is, however, relatively narrow compared to several other endoglycosidases applied for *N*-linked glycan release and it is not commercially available.

Four oligosaccharide chain-cleaving enzymes have been identified and purified to homogeneity from cultural filtrates of *Flavobacterium meningosepticum*: endoglycosidase F₁, F₂, and F₃ and the amidase peptide-*N*⁴, *N*-acetyl- β -D-glycosaminyl-asparagine amidase (PNGase F) free from endo F and protease activity was isolated (10) and purified using direct fast protein liquid chromatography (FPLC)-controlled, hydrophobic interaction chromatography of the cultural filtrate on TSK-butyl and TSK-phenyl resins, followed by FPLC-developed, high-resolution sulfopropyl chromatography in >50% yield (11). Endo F₂ and endo F₃ were shown to represent new distinct endoglycosidases that prefer complex as compared to high-mannose asparagine as do glycans. Preliminary evaluation of the substrate specificity of these enzymes indicates that F₂ cleaved biantennary oligosaccharides, whereas endo F₃ cleaved both bi- and triantennary oligosaccharides. Preparation of endoglycosidase from *Flavobacterium meningosepticum* are currently commercially available, generally being sold as "endoglycosidase F". These preparations are primarily endo F₁, with relatively small and variable amounts of endo F₂ and endo F₃ being present (12). Commercial suppliers include Europa Bioproducts, Ely, Cambridge, UK; Boehringer Mannheim (Indianapolis, IN); and Genzyme (Boston, MA).

Endoglycosidase H is in widespread use in glycoprotein research. This enzyme is active on *N*-linked glycoprotein oligosaccharides and is highly stable. It is able to release all high-mannose type oligosaccharides from glycoproteins, as well as most hybrid types of oligosaccharides (13–15). The utility of endo H is perhaps best realized in working with glycoprotein substrates that are known not to contain complex oligosaccharides (15). Endo H is commercially available from a number of sources, including Boehringer Mannheim (Indianapolis, IN) and; Genzyme (Boston, MA).

A variety of eukaryotic endoglycosidases specific for *N*-linked glycoprotein oligosaccharides have also been reported (3). The cellular slime mold *Dictyostellium discoideum* produces an endoglycosidase, termed endo S (16). Several fungal endoglycosidases were isolated from *Mucor heimalis*, termed endo M (17), and *Sporotrichum dimorphosporum*, termed endo B (18). Endo M and endo B are similar to endo F₂: they cleave high-mannose and some complex type oligosaccharides. Endoglycosidases have been also reported in higher plants and mammals (19–21).

The endoglycosidases capable of cleaving glycoprotein glycans at the *N*-acetylglucosamine–asparagine linkage are less susceptible to the steric hindrance sometimes found for glycoamidase (3). The endoglycosidases described in the foregoing cannot cleave tetraantennary glycans. To hydrolyze this class of *N*-linked oligosaccharide a different type of glycan releasing enzyme, the glycosamidases, may be applied (22). These enzymes cleave *N*-linked oligosaccharides directly between the asparaginyl residue and the reducing end *N*-acetylglucosamine residue of the glycan by cleavage of the amide linkage. In this reaction, the asparaginyl residue is converted to an aspartyl residue with consequent oligosaccharide releasing in the form of glycosylamine. These enzymes are known as glycopeptidases, peptide: *N*-glycosidases, PNGases, *N*-glycohydrolases, and *N*-glycanases. They are very similar in their substrate specificity, as they cleave all types of *N*-linked glycoprotein oligosaccharides—high-mannose, hybrid, and complex. Enzymatic release of oligosaccharides also provides the possibility to recover the protein part and to use this for analysis of the material, such as biological activity of deglycosylated protein, its functioning, and other properties (26).

The release of *O*-linked carbohydrates using enzymes is problematic. The enzymes will cleave only the disaccharide Gal- β -1,3-GalNAc *O*-linked structures (23,24). This structure can be cleaved by endo-GalNAc-ase D and endo-GalNAc-ase A, but these enzymes are active only toward the unmodified disaccharide and will not cleave the sialylated derivatives that are commonly found. *O*-linked oligosaccharides are quite variable in structure and are often extended with additional sugars (23–25) and therefore the use of the current so-called “*O*-glycanase” is of very limited value.

1.1. Points to Consider—Chemical Release

1. Chemical release will generally degrade the protein.
2. Release is frequently affected by salts and detergents.
3. Chemicals may be hazardous to handle.
4. The removal of byproducts may be difficult and samples generally require cleanup before analysis.
5. Chemical methods require specialized apparatus and knowledge.
6. The use of highly reactive material is required and may cause modification of the released glycan.

1.2. Points to Consider—Enzymatic Release

1. There may be problems of steric hindrance in the reaction of the enzyme with the glycoprotein.
2. Denaturation may be required and detergents can often interfere with subsequent labeling and analysis.
3. Selective release of more accessible glycans can occur.

4. Release may be more efficient with glycopeptides.
5. Efficient techniques for separation of the deglycosylated protein from the glycans must be used.
6. Frequently the deglycosylated protein is much less soluble and may precipitate.

2. Materials

2.1. Enzymatic Release by PNGase F and In Gel Release

1. Plastic 0.5- and 1.5-mL Eppendorf tubes washed in distilled water and dried.
2. PNGase F (either recombinant or natural form) 100 U/mL in 20 mM NaHCO₃, pH 7.0 (*see Note 1*). (This enzyme is frequently referred to as *N*-Glycanase, a trademark of Glyko Inc., Novato.)
3. 50 mM Ammonium formate, pH 8.6 (prepare by adding formic acid to ammonium hydroxide—titrate to pH 8.6), 0.4% SDS.
4. 1.2% (CHAPS), 0.1 M EDTA (add 2% dithiothreitol [DTT] before use).
5. Distilled water (*see Note 2*).
6. 20 mM NaHCO₃, pH 7.0.
7. 45 mM DTT.
8. 100 mM Iodoacetamide.
9. Dowex AG50X12 (H⁺ form) (*see Note 3*).
10. Biogel P2 gel-filtration resin (Bio-Rad, Hercules, CA).
11. Microcon 10 concentrator (Amicon, Beverly, MA).
12. Toluene.

2.2. Endoglycosidase Release

1. 1 U/mL Endoglycosidase H (*see Note 4*) in 50 mM sodium phosphate buffer, pH 7.0.
2. 20,000 mU/mL Endoglycosidase F₁ (*see Note 5*) in 50 mM sodium acetate buffer, pH 6.0.
3. 50 mM Sodium citrate phosphate buffer, pH 7.0; 25 mM EDTA, 0.1% sodium azide.
4. 1.0 M Sodium acetate buffer, pH 6.0 (stock diluted 1:5 v/v to 200 mM).
5. Protein binding membrane.

2.3. Hydrazinolysis

1. Dialysis membranes, 10,000 mol wt cutoff (Gibco-BRL, Bethesda, MD).
2. Trifluoroacetic acid (TFA).
3. Anhydrous hydrazine (Ludger Ltd., Oxford, UK) (*see Note 6*).
4. Toluene.
5. Sodium hydrogen carbonate.
6. Acetic anhydride.
7. Chromatography paper.
8. Butanol–ethanol–water mixture (4:1:1, by vol).
9. Butanol–ethanol–water mixture (8:2:1, by vol).
10. Polytetrafluoroethylene (PTFE) filters.

2.4. Automated Hydrazinolysis

1. GlycoPrep 1000 automated hydrazinolysis system (Oxford GlycoSciences Ltd., Abingdon, UK).
2. Reagents and column sets (Oxford GlycoSciences Ltd., Abingdon, UK).
3. Rotavap or equivalent drying system for drying down aqueous samples ≤ 5.0 mL.

2.5. Release of GPI Anchor from Blotted Glycoproteins

1. HPLC-grade water.
2. Screw-top Eppendorf tubes.
3. 0.5-mL Microtubes (BDH-Merck, Poole, UK).
4. 48% Aqueous hydrofluoric acid (HF), Aristar-grade (BDH-Merck, Poole, UK). Store in 0.5-mL aliquots in Eppendorf tubes at -20°C . **Caution:** Highly corrosive.
5. Dewar container.
6. Access to a freeze-drying apparatus.
7. Access to a sonicating waterbath.
8. 0.3 M Sodium acetate buffer, pH 4.0. Prepare by titrating 0.3 M sodium acetate solution to pH 4.0 with glacial acetic acid. Stable at room temperature for several months.
9. 1.0 M Sodium nitrite. Always prepare freshly just before use.
10. C8 and NH_2 Isolute™ cartridges (IST, Mid-Glamorgan, UK).
11. Methanol, HPLC grade (BDH-Merck, Poole, UK).
12. Dowex AG5OX12, 200–400 mesh (Bio-Rad, Hemel Hempstead, UK), converted to the H^+ form by washing with >10 vol 1 M HCl and >20 vol water. Store with an equal volume of water at 4°C .
13. Dowex AG3X4, 200–400 mesh (Bio-Rad, Hemel Hempstead, UK), converted to the OH^- form by washing with >10 vol 1 M NaOH and >20 vol of water. Store with an equal volume of water at 4°C .
14. Access to a SpeedVac or rotary evaporator.
15. Signal™ 2-AB labeling kit (Ludger Ltd., Oxford, UK).
16. Access to a heating block.
17. 3MM Whatman paper.
18. Small chromatography tank with rack.
19. Butan-1-ol–ethanol–water mixture (4:1:1, by vol). The paper chromatography tank should be lined with 3MM Whatman paper with some of the solvent in the bottom.
20. Access to a fume cupboard.
21. Long-wave ultraviolet (UV) lamp.
22. Access to a microcentrifuge.
23. Microcentrifuge filters (Sigma, Poole, UK).
24. 30% Acetic acid in water.
25. Acetonitrile, Aristar grade (BDH, Poole, UK).
26. Access to an SDS-PAGE system.
27. Polyvinylidene fluoride (PVDF) membrane (Amersham, Buckinghamshire, UK).

28. Access to a blotting apparatus (e.g., a semidry blotting apparatus from Hoefer Scientific Instruments, CA).
29. Amido black (Sigma, Poole, UK).
30. Razor blade.
31. Fluorescence detector (e.g., Gilson Model 121).
32. Access to a Microbore HPLC system, e.g., an Ultrafast Microprotein Analyzer (Michrom Bio Resources, CA).
33. Liquid nitrogen.
34. Powder-free gloves.
35. Dextran, grade C (BDH-Merck, Poole, UK).

3. Methods

3.1. PNGase F Release and Recovery

Suitable for analysis of *N*-linked glycans where sufficient material is available for optimization.

3.1.1. Preparation of Glycoprotein for Release by PNGase F (Method 1)

Method using denaturation—use this if in doubt about complete release:

1. Isolate the glycoprotein according to your usual procedures.
2. The sample should be relatively salt-free and contain no extraneous carbohydrates (e.g., Sephadex-purified material contains large amounts of glucose) (*see* **Notes 7–9**).
3. If the volume of the glycoprotein solution required is >100 μL , dry the glycoprotein in a 1.5-mL microcentrifuge tube. Generally 50–200 μg of glycoprotein is required.
4. The incubation of a control glycoprotein with known glycosylation alongside experimental samples is recommended. Suitable proteins for this purpose are bovine serum fetuin, ribonuclease B, or haptoglobin (*see* **Note 10**).
5. Proceed with the enzymatic digestion as described in **step 7**.
6. Store remaining glycoprotein at 4°C for future use.
7. Dissolve sample in 50 μL 50 mM ammonium formate, pH 8.6; 0.4% SDS.
8. Incubate for 3 min at 100°C.
9. Cool and add 50 μL of CHAPS detergent buffer.
10. Add 0.2 U (2 μL) of PNGase F.
11. Incubate for 24 h at 37°C (add 5 μL of toluene to prevent bacterial growth).
12. Remove 5 μL and analyze the reaction mixture by SDS-PAGE (*see* **Note 11**).
13. If sample is completely deglycosylated proceed with **step 14** otherwise continue with incubation.
14. Filter samples through protein binding membrane or perform gel filtration (*see* **Note 12**).
15. Dry sample in a rotary evaporator.

3.1.2. N-Linked Oligosaccharide Release by PNGase F (Method 2)

Method without denaturation—only use if complete release has been confirmed.

1. Dry pure, desalted glycoprotein into a 2-mL screw-top Eppendorf tube.
2. Suspend glycoprotein in 200 μ L of PNGase F digestion buffer.
3. Add 1 U/0.5 mg of glycoprotein of PNGase F (*see Note 13*) and 5 μ L of toluene (to prevent bacterial growth).
4. Incubate at 37°C for up to 72 h.
5. Centrifuge the sample briefly, and transfer to a Microcon 10 concentrator.
6. Centrifuge the concentrator at 14,000g for 20 min to separate protein and oligosaccharide components.
7. Transfer the filtrate to a 2-mL Bio-Gel P2 column equilibrated with water in a glass Pasteur pipet (*see Note 14*).
8. Elute oligosaccharides with 800 μ L of HPLC grade water (*see Note 8*).
9. Dry desalted oligosaccharides for further analysis.

3.1.3. Release from Polyacrylamide Gels with PNGase F

Suitable for analysis of low microgram amounts of protein or for unpurified proteins separated by SDS-PAGE or two-dimensional electrophoresis.

1. Run gel and remove top glass plate.
2. Cut out gel pieces with band of interest using a washed scalpel blade, keeping the piece as small as possible.
3. Put into 1.5-mL tubes and wash with 1 mL of 20 mM NaHCO₃, pH 7.0, twice using a rotating mixer, leaving for 30 min. Discard the washings.
4. Add 300 μ L NaHCO₃, pH 7.0.
5. Add 20 μ L of 45 mM DTT.
6. Incubate at 60°C for 30 min.
7. Cool to room temperature and add 20 μ L of 100 mM iodoacetamide.
8. Incubate for 30 min at room temperature in the dark. Discard solution.
9. Add 5 mL of 1:1 acetonitrile–20 mM NaHCO₃ pH 7.0.
10. Incubate for 60 min to wash out reducing agents and SDS.
11. Cut gel into pieces of 1 mm².
12. Place in SpeedVac to dry.
13. Add 30 μ L (3 U) of PNGase F in 20 mM NaHCO₃, pH 7.0
14. Allow gel to swell and then add a further 100 μ L aliquot of buffer.
15. Incubate at 37°C for 12–16 h.

3.2. Endoglycosidase Release

Suitable for selective release of different classes of *N*-linked glycans.

1. Prepare solution of glycoprotein.
2. Make up 20,000 mU/mL of endoglycosidase F₁ or 1 U/mL of endo glycosidase H in appropriate buffer.
3. For incubation with endoglycosidase F₁ add 200 mU of enzyme solution (10 μ L).
4. For incubation with endoglycosidase H add 40 mU (25 μ L) of enzyme solution.
5. Incubate at 37°C for 18 h.
6. Pass through a protein binding membrane.
7. Evaporate to dryness.

3.3. Hydrazinolysis

Suitable for analysis of *N*- or *O*-linked glycans in which the amount of protein is limited, steric hindrance to enzymatic release is known, or selective release of glycans by enzymatic means is suspected.

3.3.1. Preparation of Samples for Hydrazinolysis

1. Desalt the samples completely.
2. Dissolve the sample in 0.1% TFA in as small a volume as possible.
3. Set up dialysis at 4°C (*see Note 15*).
4. Dialyze for a minimum of 48 h.
5. Recover sample from dialysis membrane. Wash membrane with 0.1% TFA to ensure recovery.
6. Transfer to a suitable tube for hydrazinolysis.
7. Lyophilize the sample.
8. For *O*-glycan analysis further drying is recommended.
9. Remove sample from the lyophilizer immediately prior to addition of hydrazine.

3.3.2. Manual Hydrazinolysis Procedure

Suitable for analysis of *N*- and *O*-linked glycans when expertise and equipment for procedure are available.

1. Place sample (dialyzed against 0.1% aqueous TFA) in acid-washed glass tube.
2. Completely lyophilize the sample for 2 d.
3. Remove tubes from drying immediately prior to hydrazine addition.
4. Flush tube with argon, taking care not to dislodge lyophilized protein.
5. Rinse a dried syringe fitted with a stainless steel needle with anhydrous hydrazine and discard the liquid.
6. Take up fresh hydrazine and dispense onto the sample; 0.1 mL hydrazine is sufficient to dissolve up to 2 mg of glycoprotein. For larger amounts add more hydrazine.
7. Seal the tube.
8. Gently shake tube—the protein should dissolve.
9. Place in an incubator (use water bath).
10. For release of *N*-linked glycans incubate at 95°C for 5 h, for *O*-glycan release incubate for 60°C for 6 h.
11. Allow to cool and remove hydrazine by evaporation.
12. Add 250 μ L toluene and evaporate; repeat 5 times.
13. Place tube on ice and add 100 μ L saturated sodium bicarbonate solution.
14. Add 20 μ L acetic anhydride.
15. Mix gently and leave at 4°C for 10 min.
16. Add a further 20 μ L acetic anhydride.
17. Incubate at room temperature for 50 min.
18. Pass solution through a column of Dowex AG50X12 (H⁺ form)—0.5 mL bed volume.
19. Wash tube with 4 \times 0.5 mL water and pass through a Dowex column.
20. Evaporate to dryness. This should be done in stages by redissolving in decreasing volumes of water.

21. Prepare a 80×2 cm strip of chromatography paper (prewashed in water by descending chromatography for 2 d).
22. Spot sample on strip, and perform descending chromatography in 4:1:1 Butanol-Ethanol-water for 3 d (*N*-glycans) or 8:2:1 Butanol-Ethanol-water for 2 d (*O*-glycans).
23. Elute region from -1 cm to $+3$ cm of origin with 4×0.5 mL water. Filter through PTFE filter and dry.

3.3.3. Automated Hydrazinolysis (27)

Suitable for the routine release of *N*- or *O*-linked glycans, e.g., for quality control and where a high degree of reproducibility between samples is required.

1. Prepare samples by dialysis against 0.1 *M* TFA at 4°C.
2. Transfer samples to GlycoPrep reaction vials (see **Note 17**).
3. Lyophilize the sample in reaction vials.
4. Set up GlycoPrep 1000 with new column sets and collection tubes.
5. Select the desired program.
6. Load samples from the lyophilizer.
7. Start the instrument run.
8. Samples in collection vials may be removed when the system wash protocol commences (see **Note 18**).
9. Dry the sample down on Rotavap or similar apparatus. It is necessary to divide the sample into smaller aliquots for drying in centrifugal evaporators.
10. The sample is then ready for direct analysis or for derivatization.

3.3.4. Small Scale GPI Release and 2-AB Labeling “On the Blot”—Procedure of Zitzmann and Ferguson (28)

Suitable for the analysis of GPI anchors attached to proteins separated by SDS-PAGE.

1. Apply 5 μ g of protein (or an equivalent of at least 100 pmol) on a 10% polyacrylamide gel and subject to SDS-PAGE.
2. Transfer proteins from the gel to a PVDF membrane by electroblotting.
3. Stain the PVDF membrane with amido black, cut out the protein bands of interest using a razor blade, and transfer into screw-top Eppendorf tubes.
4. Deaminate the samples by completely submerging the blot strips in 50 μ L of 0.3 *M* NaAc, pH 4.0, and 50 μ L of freshly prepared 1 *M* sodium nitrite.
5. Wash the strips three times with water to remove salt, transfer into 0.5-mL Eppendorf tubes and dry.
6. Prepare 2-AB labeling reagent as described by manufacturer.
7. Take care to completely wet each blot strip with the labeling reagent (usually 15 μ L are sufficient), cap the tubes, and label the strips for 2–3 h at 65°C in a heating block.
8. Wash the blot strips three times with about 10 mL of 50% acetonitrile, transfer to screw-top Eppendorf tubes, and dry.
9. Add 40 μ L (or as much as needed to submerge the strip) of ice-cold 48% aqueous HF acid and dephosphorylate the samples by leaving them for 60–72 h on ice-water.

10. Remove the HF by freeze-drying. Add 100 μL of water to each tube and freeze-dry again. Repeat this step.

4. Notes

1. Peptide *N*-glycosidase F is available from Sigma, Poole, UK. It is advisable to use a glycerol-free preparation of the enzyme, as this can interfere with subsequent fluorescence labeling reaction efficiencies.
2. For preparations in which analysis is to be performed with mass spectrometry, particularly matrix-assisted laser desorption–time-of-flight (MALDI-TOF), glass-distilled water should be used as deionized water may contain polymeric material which will interfere with the analysis.
3. Dowex AG50 \times 12 (200–400 mesh) should be used for desalting of glycans as there will be minimal loss on this grade. It may be prepared in the H^+ form by washing with 10 vol of 1 *M* HCl followed by water until the pH is the same as that of the wash water (usually slightly acidic).
4. Endoglycosidase H from *Streptomyces plicatus* is available from Sigma, Poole, UK. The specificity of this enzyme is that it will cleave the chitobiose core structure of oligomannose or hybrid type glycans attached to the asparagine residue of a glycopeptide. The presence of 1, 6-linked fucose on the *N*-acetylglucosamine linked to the peptide will not affect activity. The free glycan or the dolichol pyrophosphate derivative will also be cleaved.
5. Endoglycosidase F is available from Europa Bioproducts, Ely, Cambridge, UK. Three different types of endoglycosidase F with distinct specificities have been cloned. The general specificity is such that it will cleave the chitobiose core of oligomannose or hybrid type glycans but will not cleave complex type glycans. The presence of fucose 1, 6-linked to *N*-acetylglucosamine attached to the peptide will reduce the rate of cleavage >50 times.
6. Hydrazine is toxic and flammable; discard ampoule and residual contents after using once. Dispose of safely according to your institution's regulations.
7. The glycoprotein sample should ideally first be dialyzed against distilled water and stored lyophilized in a 1.5-mL microfuge tube. If the sample needs to be in a buffered solution, one can place it in 50 *mM* sodium phosphate buffer, pH 7.5, at a final concentration of at least 100 $\mu\text{g}/\mu\text{L}$ or 2 *mg/mL*. Best results are obtained if the total salt concentration of the solution is >100 *mM*. The use of a Tris-based buffer is not recommended. If desired, the sample may also contain 0.05% sodium azide.
8. It is recommended that at least 250 μg of glycoprotein is used for analysis.
9. The actual amount of glycoprotein required for profiling will depend on the size of the protein, the amount of glycosylation, and the degree of oligosaccharide heterogeneity. In general, the amount of glycoprotein required increases with the size of the protein or the degree of heterogeneity and decreases with the percent of glycosylation. As a general guideline, one would start with approx 50–100 μg to profile the *N*-linked oligosaccharides of a 60-kDa glycoprotein that contains 10–20% carbohydrate by weight. For *O*-linked oligosaccharide analysis we suggest 100–500 μg of starting glycoprotein. This amount would normally provide sufficient material for several electrophoretic runs. For isolation of individual oli-

- gosaccharides, and carrying out sequencing, additional material is required.
10. A suitable control in which *N*-glycans have been well characterised should be used as a control for enzyme digestion. Examples of such glycoproteins include ribonuclease B, bovine serum fetuin, and haptoglobin (all available from Sigma–Aldrich but there may be some variation in glycosylation profile between different batches). Use of the control ensures that the reagents are working and that release and labeling procedures were performed properly.
 11. It is advisable to assay the degree of glycosylation both before and after PNGase F digestion by SDS-PAGE.
 12. Extra care should be taken to thoroughly desalt denatured samples to ensure that subsequent fluorescence labeling reactions are not affected. Protein components can also be removed by precipitation with ice-cold ethanol. 75% v/v
 13. Deglycosylation can also be performed using PNGase A. But in this case the protein should be cleaved to glycopeptides by appropriate protease enzymes. This is especially relevant if plant glycoproteins are being studied, as PNGase F will not cleave oligosaccharides with core fucose residues in α 1–3 linkage.
 14. Bio-Gel P2 has an exclusion limit of approx 1.8 kDa. If a 2-mL column has an exclusion volume of about 600 μ L, then elution of the column with 800 μ L of water should elute all *N*-linked oligosaccharides. If in doubt, fractions can be assayed for hexose using the phenol–sulfuric acid method.
 15. Dialysis against 0.1% TFA will remove most salts and detergents but should always be performed in the cold (about 4°C) to minimize desialylation under the acidic conditions. In certain cases this may not be feasible, however (e.g., if protein precipitates) and in these situations purification by reverse-phase chromatography in volatile solvents that may be removed under vacuum is recommended.
 16. Protein samples for hydrazinolysis should be essentially salt free. Salts, heavy metal ions, dyes, and detergents may interfere with the hydrazinolysis reaction in an unpredictable way and need to be removed. Unless the sample has been desalted by other techniques such as reverse-phase chromatography (*see Note 15*) this is most conveniently performed by dialysis.
 17. The maximum amount of sample is 2.0 mg. The minimum amount of sample depends on the glycoprotein and analytical technique to be used but is generally in the range of 10–100 μ g.
 18. All chemistry has been completed by this step and the system is being washed before the next run. The sample is delivered in dilute acetic acid (approx 5.0 mL). If it is left in this solution at elevated temperatures there is a possibility of desialylation of the released glycans and the sample should therefore be dried down as soon as possible after collection.

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