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Chemical Deglycosylation Kit

Instruction Manual

Catalog # 170-6492

For technical service, call your local Bio-Rad office, or
in the US, call 1-800-4BIORAD (1-800-424-6723)



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Section 1

Kit Specifications and Components

Application

The chemical deglycosylation of glycoproteins.

Contents

Sufficient reagents and reaction vials for chemical deglycosylation of up to 10 x 1 mg of glycoprotein.

Starting Material

Dry, salt-free glycoprotein or glycopeptide.

Storage

This kit should be stored at 4°C immediately upon receipt.

Performance

Polypeptide recovery typically 90%

Polypeptide cleavage <10%

Extent of deglycosylation >80%

Kit Components

- 10 reaction vials, caps and PTFE-faced seals.
- 2 vials of reagent A, contains TFMS, trifluoromethanesulfonic acid, a highly corrosive acid; should be stored at 4°C or -20°C in the dark until use (see MSDS for proper disposal and safety measures).
- 2 vials of reagent B, contains toluene, a volatile organic solvent; store at 4°C (see MSDS for proper disposal and safety measures).
- 2 vials of reagent C, contains pyridine, a volatile organic solvent; store at 4°C (see MSDS for proper disposal and safety measures).

Section 2 Introduction

Introduction

A wide variety of eukaryotic membrane-bound and secreted proteins are glycosylated. In addition, certain intracellular eukaryotic proteins are glycoproteins (Montreuil 1982). Glycosylation of proteins in eukaryotes occurs in three ways (Parekh et al. 1989). Glycosylation through a glycosidic bond to an asparagine side chain is known as N-glycosylation. Such asparagine residues only occur in the amino acid sequence of Asn-xaa-Ser/Thr where xaa can be any amino acid. The carbohydrate portion of a glycoprotein is also known as a glycan. O-glycans are linked to serine or threonine side chains through O-glycosidic bonds. The polypeptide may be linked to a phosphatidylinositol lipid anchor through a carbohydrate bridge, the whole assembly being known as a glycosyl-phosphatidylinositol (GPI) anchor (Ferguson and Williams 1988).

In recent years, the functional significance of the carbohydrate moieties has been increasingly appreciated (Rademacher et al. 1988). Carbohydrates covalently

attached to polypeptide chains can confer many functions to the glycoprotein, for example resistance to proteolytic degradation, the transduction of information between cells, and intercellular adhesion through ligand-receptor interactions (Ashwell and Hartford 1982, Dennis et al. 1987, Gesundheit et al. 1987, Podskalny et al. 1986). With the increase in understanding of carbohydrate functions, the need for rapid, reliable, and sensitive methods for carbohydrate detection has grown considerably.

The presence of carbohydrates can often complicate structural characterization of the polypeptide moiety of a glycoprotein. Removal of the carbohydrate from the polypeptide portion of a glycoprotein, leaving the polypeptide intact, can facilitate further analysis of the polypeptide without further interference from the carbohydrate. Comparative analysis of the glycoprotein before and after glycosylation allows the relative contributions of carbohydrate and protein to the overall molecular size to be determined, and can also be used to obtain some information regarding the functional significance of the carbohydrate portion of the molecule.

Applications (Leavitt et al. 1977, Sojar and Bahl 1987, Trimble and Maley 1977) of glycoprotein deglycosylation have centered around:

- A determination of the extent of glycosylation of the glycoprotein and an estimation of the molecular weight of the polypeptide moiety
- The preparation of peptides and polypeptides for primary structural analysis
- Studies on the role of carbohydrates in the functional properties of glycoproteins

This protocol has been optimized, using a broad range of glycoproteins, with respect to reaction temperature, incubation time, scavenger, and the amount of reagent required to deglycosylate up to 1 mg of glycoprotein.

Standard glycoproteins used to optimize the protocol include bovine serum fetuin, human α 1-acid glycoprotein, horseradish peroxidase, hen egg ovalbumin, and bovine pancreatic ribonuclease B. The protocol has the following steps:

1. Sample preparation
2. Deglycosylation
3. Neutralization of excess reagent
4. Recovery of deglycosylated polypeptide

Little information is available on the exact mechanism of TFMS deglycosylation. It is known that the reaction occurs under anhydrous conditions, which is indicative of deglycosylation via acid catalyzed dehydration. A scavenger species is employed (reagent B) to neutralize reactive groups formed during the deglycosylation reaction, which may otherwise attach to the polypeptide portion of the molecule. The neutralization of TFMS is accomplished by adding reagent C, which results in the formation of the pyridinium salt.

Required reagents, not supplied in the kit

- 0.5% (w/v) ammonium bicarbonate
- Dry ice
- Ethanol (50–100 ml)
- A clean, dry glass syringe and needle (recommended volume of 50–200 μ l)

Section 3 Special Precautions

Please read before proceeding to actual protocol

- The protocol has been optimized using a broad range of standard glycoproteins and is generally applicable, but may not work for all glycoproteins. Certain glycoproteins may be unexpectedly sensitive or resistant to deglycosylation by TFMS. If this situation is suspected, a time course analysis to monitor the extent of deglycosylation should be performed.
- Reagent A is a very powerful acid and should be handled in a fume hood cupboard while wearing eye protection and gloves. (See MSDS for detailed precautions and disposal information.) This reagent is hygroscopic; a fresh vial should be used for each batch of glycoproteins treated and the reagent should be discarded after it has been exposed to the atmosphere for over 5 min.
- An ethanol/dry ice bath is needed prior to addition of TFMS and during the neutralization step.

Section 4

Protocol

Step 1: Sample Preparation

1. It is important that the sample is free of salts, metal ions, and detergents. This can be achieved by dialysis against an appropriate solvent (e.g., 0.1% w/v trifluoroacetic acid), gel filtration, or reverse phase HPLC in a volatile system. **Note:** The TFMS reaction has a tolerance for the presence of small amounts of SDS in the sample; this tolerance may, however, be sample dependent and it is recommended to keep the SDS concentration to a minimum.
2. Transfer the aliquot of salt-free glycoprotein in solution (≤ 0.5 ml) to the bottom of a reaction vial and freeze-dry thoroughly (see also 3 below). Tightly seal the vial using the PTFE-lined septum and cap.
3. It is important that the sample is thoroughly dry when the reagents are added to initiate the deglycosylation reaction. Therefore, care should be taken that the drying step reaches completion and that the dehydrated samples are not exposed to moisture

before the deglycosylation step. Lyophilization of the glycoprotein is recommended (≤ 0.5 mTorr, >24 hr).

Step 2: Deglycosylation

1. Prepare a dry ice/ethanol bath.
2. Score the TFMS vial before opening. Tap the vial gently to ensure the contents are at the bottom. Open slowly and add 60 μ l of reagent B using a clean dry syringe. Shake gently. Rinse the syringe with the mixture.
3. Place the capped vial containing the dry sample into the ethanol/dry ice bath so that it rests on the ice and on the side of the vessel. Allow vial to cool for 20 sec.
4. Using the syringe, add 50 μ l of the yellow reagent mixture to each sample by piercing the PTFE-lined septum of the vial with the syringe needle and then allowing the reagent to run slowly down the side of the reaction vessel over a period of 15–20 sec. Do not be concerned if some of the reagent freezes on the side of the reaction vessel. Withdraw the needle and leave the vial in the reaction bath for another 10 sec. **Note:** when piercing the septum with a syringe needle, precautions should be taken to

avoid risk of personal injury. The use of a remote handling device is recommended to hold the vial during needle insertion. Alternatively, hand protection should be worn during this procedure.

5. Place vial in freezer at -20°C . After 5 min, remove from freezer and shake well to aid melting and subsequent solvation of glycoprotein. Place vial in freezer for another 5 min and shake again. The contents should now appear melted and be homogeneous. Place vial back in freezer for 4 hr.

Step 3: Neutralization of Excess TFMS

1. Remove the reaction vial from freezer and remove the septum from inside of the cap. Place the vial in the ethanol/dry ice bath as before and allow to cool for 20 sec.
2. Using the micropipet, slowly add 150 μl of reagent C to the vial by allowing the reagent to flow down the side of the reaction vessel over a period of 15–20 sec. Leave the vial in the bath for another 20 sec.
3. Transfer the vial to dry ice for 5 min and then to wet ice for a further 15 min.

4. Add 400 μl of 0.5% ammonium bicarbonate to the vial and mix briefly. A precipitate may form in the sample at this point.

Step 4: Recovery of Deglycosylated Polypeptide

After deglycosylation, the polypeptide must be isolated from the reagents and from the other reaction products. Separation is usually accomplished on the basis of molecular size using methods such as gel filtration and dialysis. With both methods, care should be taken to choose an appropriate equilibration buffer. See below for detailed descriptions of peptide recovery.

Dialysis

The recommended procedure for isolation of a deglycosylated peptide from the neutralized reaction mixture using dialysis is as follows:

Transfer all or some of the neutralized reaction mixture to which ammonium bicarbonate buffer has been added to a dialysis vessel. If batch dialysis is used, dialyze against a minimum of 3 x 1,000 volumes of ammonium bicarbonate buffer. A minimum of 2 hr dialysis time is recommended for each batch. If flow dialysis is used, dialysis against 500–1,000 volumes of buffer is recommended over a period of at least 8 hr.

Gel Filtration

In cases where no precipitation of the peptide is observed on addition of the aqueous buffer to the neutralized reaction mixture, it may be possible to isolate the peptide by passing the reaction mixture through a small gel filtration column.

Commercially available gel filtration columns can be chosen according to the molecular weight of the polypeptide to be recovered.

Precipitation

In cases where precipitation is observed on addition of the aqueous buffer, it may be possible to isolate the precipitated peptide directly by centrifugation. The recommended procedure is as follows:

1. Cool the sample to 4°C and allow to stand at this temperature for 30 min.
2. Centrifuge at high speed for 10–15 min at 4°C to sediment precipitated material.
3. Decant and save the supernatant. Resuspend pellet in a small volume of ice-cold buffer and recentrifuge at 4°C.
4. Repeat step 3 at least three more times.

Deglycosylation of Mucins

The removal of glycans from mucins is more difficult than from glycoproteins. In fact, the innermost GalNAc is often unaffected by TFMS.

The following method has been shown to work for this application.

1. Deglycosylate protein as described in step 2 of the standard protocol.
2. After neutralization dialyze the sample against 50 mM Tris-HCl, pH 7.5, 100 mM NaCl.
3. Add periodate to 10 mM and incubate for 6 hr at 5°C.
4. Dialyze against water and lyophilize in preparation for a second deglycosylation.
5. Deglycosylate for a second time as described in step 2 of the standard protocols.

Section 5 Troubleshooting Guide

Problem	Probable Cause	Recommended Solution
1. Deglycosylation is incomplete	a) Sample not sufficiently dry prior to deglycosylation.	Ensure that sample is lyophilized for a minimum of 24 hr (50 mTorr) and avoid extended exposure to atmosphere between drying and deglycosylation stages
	b) Reagent A was exposed to atmosphere for excessive length of time prior to addition of reagent to sample	Ensure that all samples to be deglycosylated are conveniently placed so that addition of reagent A to successive samples may occur without delay

Problem	Probable Cause	Recommended Solution
	c) Sample and reagents not homogenized sufficiently	On inspection of sample, shake well and swirl the liquid contents around the side of the vessel until all solid material is washed to the bottom
	d) Deglycosylation conditions are not optimal	The conditions described in this protocol have been optimized using a range of standard glycoproteins. It is possible that a particular glycoprotein may be resistant to deglycosylation by reagent A. If this is suspected, then a time course analysis of the extent of deglycosylation can be performed. A suitable time range may be 4–8 hr.

Problem	Probable Cause	Recommended Solution
2. Peptide damage is apparent on analysis after deglycosylation	a) Failure to cool samples sufficiently prior to TFMS addition or neutralization	Ensure that at least one third of the reaction vessel is submerged in a dry ice/ethanol bath for a minimum of 20 sec prior to slow addition of reagent A
	b) Deglycosylation conditions are not optimal; see 1. d) above.	
3. Low recovery of polypeptide	a) Gel filtration — Precipitation of polypeptide on passage through gel filtration column	Use alternative method for recovery
	b) Precipitation — Partial solvation of sedimented precipitate on washing with cold buffer	Retain and pool all washes plus supernatant from the initial centrifugation. Evaporate and reconstitute with buffer.

Problem	Probable Cause	Recommended Solution
c) Dialysis — Failure to recover material from dialysis chamber or bag	Rinse dialysis chamber or bag at least twice with buffer after dialysis is complete. This is particularly important if signs of precipitation are apparent during dialysis.	

Section 6 References

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- Rademacher TW et al., Glycobiology, *Ann Rev Biochem* 57, 785–838 (1988)
- Sojar HT and Bahl OP, Chemical deglycosylation of glycoproteins, *Methods Enzymol* 138, 341–350 (1987)
- Trimble RB and Maley F, Subunit structure of external invertase from *Saccharomyces cerevisiae*, *J Biol Chem* 252, 4409–4412 (1977)

Section 7 Hazard Risks and Warnings



Highly flammable. Toxic by inhalation, in contact with skin and if swallowed. Causes burns. Danger of very serious irreversible effects. Do not breathe gas, fumes, vapor, mist, or spray. Avoid contact with eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Take off immediately all contaminated clothing. After contact with skin, wash immediately with plenty of water. Do not empty into drains. Take precautionary measures against static discharges. Wear suitable protective clothing, glove and eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately. Show label. This material and/or its container must be disposed of as hazardous waste.

Leichtentzündlich. Giftig beim Einatmen, Verschlucken und Berührung mit der Haut. Verursacht Verätzungen. Ernste Gefahr irreversiblen Schadens. Gas/Rauch/Dampf/Aerosol

nicht einatmen. Berührung mit den Augen vermeiden. Bei Berührung mit den Augen gründlich mit Wasser abspülen und Arzt konsultieren. Beschmutzte, getränkte Kleidung sofort ausziehen. Bei Berührung mit der Haut sofort abwaschen, mit viel Wasser abspülen. Nicht in die Kanalisation gelangen lassen. Massnahmen gegen elektrostatische Aufladungen treffen. Bei der Arbeit geeignete Schutzkleidung, Schutzhandschuhe und Schutzbrille/ Gesichtsschutz tragen. Dieser Stoff und/oder sein Behälter sind als gefährlicher Abfall zu entsorgen. Bei Unfall oder Unwohlsein sofort Arzt hinzuziehen. Wenn möglich dieses Etikett vorzeigen.

Très inflammable. Toxique par inhalation, par contact avec la peau et par ingestion. Provoque des brûlures. Danger d'effets irréversibles très graves. Ne pas respirer les gaz/vapeurs/fumées/aérosols. Eviter le contact avec les yeux. En cas de contact avec les yeux, laver immédiatement et abondamment avec de l'eau et consulter un spécialiste. Enlever immédiatement tout vêtement souillé ou éclaboussé Après contact avec la peau, se laver avec de l'eau. Ne pas jeter les résidus à l'égoût. Eviter l'accumulation de charges électrostatiques. Porter un vêtement de protection approprié, des gants et un appareil de protection des yeux/du visage. En cas d'accident ou de

malaise consulter immédiatement un médecin (si possible lui montrer l'étiquette). Éliminer le produit et/ou son récipient comme un déchet dangereux.

Facilmente infiammabile. Tossico per inalazione, contatto con la pelle e per ingestione. Provoca ustioni. Pericolo di effetti irreversibili molto gravi. Non respirare i gas/fumi/vapori/aerosoli. Evitare il contatto con gli occhi. In caso di contatto con gli occhi, lavare immediatamente e abbondantemente con acqua e consultare un medico. Togliersi di dosso immediatamente gli indumenti contaminati. In caso di contatto con la pelle lavarsi immediatamente ed abbondantemente con acqua. Non gettare i residui nelle fognature. Evitare l'accumolo di cariche elettrostatiche. Usare indumenti protettivi e guanti adatti e proteggersi gli occhi/la faccia. In caso d'incidente o di malessere consultare il medico (se possibile, mostrargli l'etichetta). Questo materiale e/o il suo contenitore devono essere smaltiti come rifiuti pericolosi.

Section 8

Ordering Information

Catalog #	Product Description
170-6492	Chemical Deglycosylation Kit
170-6490	Immun-Blot Kit for Glycoprotein Detection
170-6500	Enzymatic Deglycosylation Kit
170-6508	Deglycosylation Enhancement Kit