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Protocols in Biotechnology

Immunological Detection of Glycoproteins on Blots Based on Labeling with Digoxigenin

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ABSTRACT

In the following, we describe the application of the DIG/anti-DIG system for the (structural) analysis of glycoproteins on blots. Special emphasis is being placed on the variety of the different DIG applications in order to obtain structural information concerning the glycoprotein carbohydrate chains. Prominent among them is the use of lectins with well-known specificities for carbohydrate structures.

Index Entries: Glycoproteins; digoxigenin; immunological detection.

INTRODUCTION

The analysis of macromolecules (e.g., nucleic acids, proteins, glycoconjugates) after electrophoretic separation has become increasingly important in recent years for several reasons.

1. It combines the powerful resolution capabilities of electrophoretic systems with very sensitive detection techniques. This allows the analysis of even small amounts of macromolecules present in complex mixtures without the need of previous purifications.

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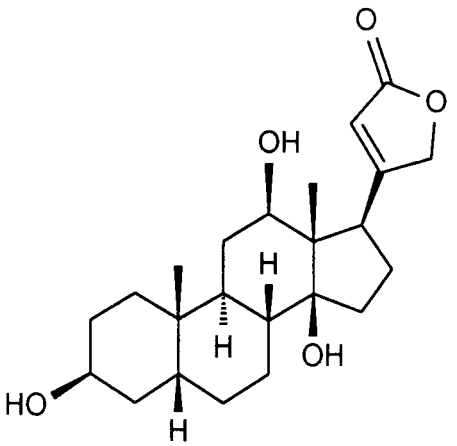

Fig. 1. Structure of digoxigenin.

2. Because of the availability of efficient blotting techniques, the application and handling of sensitive detection techniques (e.g., based on biotin/streptavidin or immunological technologies) have become possible.
3. These analytical techniques can be used without the need of expensive equipment and special knowledge (e.g., NMR or MS techniques).

In order to apply these powerful techniques to the analysis of glycoproteins, we have developed an immunological labeling and detection system. It is based on the use of the plant steroid digoxigenin as a hapten and polyclonal antibodies with high affinity toward this hapten. (*See* Fig. 1 for the structure of digoxigenin.) Such an immunological system has some advantages compared to the biotin/(strept)avidin system: (1) reduced unspecific binding behavior compared to (strept)avidin, and (2) a much lower number of binding sites for digoxigenin and absence of endogenous digoxigenin in most biological systems compared to biotin.

The basic feature of this particular system (in short: DIG/anti-DIG) consists of the labeling of the glycan part of glycoproteins with digoxigenin, either by chemical or enzymatical means (*1*), or by binding of digoxigenylated lectins (*2*). The DIG hapten is then being detected by the use of an anti-DIG conjugate, especially by employing an alkaline phosphatase (AP) conjugate producing colored products on the blots.

The combination of the different labeling and detection methods described here allows us to obtain a substantial amount of information when analyzing glycoproteins on blots. The analytical potential can be increased even further when these methods are combined with the use of exo- and endoglycosidase digestions (*3,4*). Thus, a carbohydrate detected by the general labeling method with DIG-hydrazide can be assumed to be present in a *N*-glycan linkage if there is no positive reaction after the glycoprotein

has been treated with *N*-glycosidase F, which removes all types of *N*-glycan chains. Similarly, the presence of a high mannose structure detected by a positive reaction with the lectin GNA can be corroborated by showing that it is sensitive to endo H treatment. Since lectins usually react with the terminal parts of the glycan chains, it is very often possible to combine their use with that of exoglycosidases. When the positive reaction with a lectin is no longer present after treatment with exoglycosidases, e.g., neuraminidase, α-fucosidase, β-galactosidase, or α-mannosidase, the conclusion on the structural feature present is considerably substantiated. Thus, a wealth of structural information can be obtained by combining all these different methods for the analysis of blotted glycoproteins.

The use of the DIG/anti-DIG method has further been described for the very sensitive analysis of glycolipids on TLC plates (*5*) and has been applied in combination with gold labeling for the use in histochemistry (*6*).

In the following, we describe the application of this DIG/anti-DIG system for the (structural) analysis of glycoproteins on blots. Special emphasis is being placed on the variety of the different DIG applications in order to obtain structural information concerning the glycoprotein carbohydrate chains. Prominent among them is the use of lectins with well-known specificities for carbohydrate structures.

MATERIALS

1. Digoxigenin-succinyl-ε-amino caproic acid hydrochloride: 5 m*M* solution in dimethylformamide.
2. Antidigoxigenin antibody (Fab fragments), conjugated with alkaline phosphatase (750 U/mL); available from Boehringer Mannheim (Mannheim, Germany).
3. Proteins and glycoproteins: creatinase (rec. from *E. coli*), carboxypeptidase Y, fetuin, asialofetuin, transferrin, erythropoietin (recombinant from CHO cells) from Boehringer Mannheim, and α_1-acid glycoprotein and lactoferrin from Sigma (Deisenhofen, Germany).
4. Molecular-weight markers: Trypsin inhibitor (M_r 20,100), lactate dehydrogenase (M_r 36,500), glutamate dehydrogenase (M_r 55,400), and phosphorylase b (M_r 97,400).
5. Nitrocellulose membranes (e.g., BA 85 from Schleicher & Schüll, Dassel, Germany) or PVDF membranes (e.g., Immobilon from Millipore, Eschborn, Germany).
6. Blocking reagent: casein, special quality, Boehringer Mannheim.
7. Triton X-100, Nonidet P-40, sodium dodecysulfate (SDS), octylglucoside, dithiothreitol, Tris, glycine (all p.a. grade).
8. Tris-buffered saline (TBS): 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.

9. Phosphate-buffered saline (PBS): 50 m*M* potassium phosphate, pH 7.5, 150 m*M* NaCl.
10. Buffer 1: 0.1*M* sodium acetate, pH 5.5.
11. Buffer 2: 0.1*M* Tris-HCl, pH 9.4, 0.05*M* $MgCl_2$.
12. Buffer 3: TBS, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$.
13. Buffer 4: 0.1*M* potassium phosphate, pH 6.0.
14. SDS sample buffer: 0.1*M* Tris-HCl, pH 6.8, 8% SDS (w/v), 40% glycerol (v/v), 20% 2-mercaptoethanol (v/v).
15. Sodium metaperiodate solution (15 m*M*): Dissolve 3.3 mg sodium metaperiodate (e.g., Merck No. 6597, Merck, Darmstadt, Germany) in 1 mL bidistilled water.
16. Sodium disulfite solution (20 m*M*): Dissolve 3.75 mg sodium disulfite (e.g., Merck No 6528) in 1 mL bidistilled water.
17. Blocking solution: Dissolve 0.5 g blocking reagent in 100 mL TBS by heating to 50–60°C for approx 1 h; the dissolution can be accelerated by ultrasonication and incubation in a microwave oven; the solution remains turbid.
18. 5-Bromo-4-chloro-3-indolyl-phosphate solution: Dissolve 50 mg in 1 mL dimethylformamide.
19. 4-Nitroblue tetrazoliumchloride solution: Dissolve 75 mg in 70% dimethylformamide (v/v).
20. Ponceau S solution (0.2%): Dissolve 0.2 g Ponceau S in 100 mL 3% acetic acid.
21. Enzymes (Boehringer Mannheim):
    a. Galactose oxidase;
    b. Neuraminidase (*Arthrobacter ureafaciens*);
    c. *N*-Glycosidase F;
    d. Endoglycosidase F;
    e. Endoglycosidase H.
22. Lectins, digoxigenin labeled (Boehringer Mannheim):
    a. Aleuria aurantia (AAA; specific for Fuc α1-6 GlcNAc);
    b. Concanavalin A (Con A; specific for Man/Glc);
    c. Datura stramonium (DSA; specific for Gal β 1-4 GlcNAc);
    d. Galanthus nivalis (GNA; specific for Man α1-2/3/6 Man);
    e. Maackia amurensis (MAA; specific for NeuAc α2-3 Gal);
    f. Peanut (PNA; specific for Gal β1-3 GalNAc);
    g. Phytohemagglutinin-L (PHA-L; specific for Galβ1-4GlcNacβ1-2,6Man);
    h. Ricinus communis (RCA 120; specific for β-D-Gal);
    i. Sambucus nigra (SNA; specific for NeuAc α2-6 Gal); and
    j. Wheat germ (WGA; specific for GlcNAc).
23. Standard electrophoresis apparatuses, such as the Mini systems from Bio-Rad (München, Germany) or Biometra (Göttingen, Germany) are used for SDS-PAGE; for blotting, tank blot or semi-dry blot devices are suitable. Protein separation can also

be carried out with Pharmacia Phast gels (Pharmacia LKB, Freiburg, Germany); an easy way to blot Phast gels is described by Braun and Abraham (*7*).

When using Pharmacia Phast IEF gels for the separation, these gels have to be preequilibrated for 30 min at room temperature with the following solution: 10 mL H_2O, 0.5 mL Nonidet P-40, 9 g urea, and 2 mL Pharmalyte 3-10 (in case of a pH 3–9, IEF Phast gel; Pharmacia LKB) before use to avoid high background staining in the glycan detection reaction. Other IEF gel systems can also be used for the protein separation, such as the one described in ref. (*8*). Blotting out of IEF gels is efficiently accomplished by a simple diffusion blotting procedure as described in ref. (*9*).

METHODS

Labeling Procedures Using Digoxigenin-Succinyl-ε-Amino Caproic Acid Hydrazide (DIG-Hydrazide)

General Labeling and Detection of Glycoproteins

The question of whether or not a protein contains carbohydrate or how many glycoproteins may be present in a sample can best be answered by a general, yet selective labeling procedure for carbohydrates. In this regard, periodate oxidation of vicinal diols in sugars resulting in aldehydes is the method of choice. DIG-hydrazide is then linked to these aldehyde groups. The procedure can be performed in two ways: (1) oxidation with periodate and labeling with DIG-hydrazide of the glycoproteins in solution; separation by electrophoresis and transfer onto a suitable membrane; detection of digoxigenin with a labeled anti-DIG antibody, or (2) oxidation and labeling of proteins on membranes and subsequent detection of digoxigenin. The volumes stated refer to a 50–100 cm^2 membrane; the membranes are incubated by gentle agitation at room temperature, except for color development, which should be done without shaking.

PROCEDURE 1: OXIDATION AND LABELING IN SOLUTION

1. Dissolve 0.1–10 μg glycoprotein in 20 μL buffer 1 or dilute the glycoprotein solution at least 1+1 with buffer 1 (*see* Note 1).
2. Add 10 μL of 15 m*M* sodium metaperiodate, mix, and incubate for 20 min at room temperature in the dark (*see* Note 2).
3. Destroy excess periodate by adding 10 μL of 20 m*M* sodium disulfite.

4. After 5 min at room temperature, add 5 μL of 5 m*M* digoxigenin-succinyl-ε-amino caproic acid hydrazide, and incubate the mixture for 60 min at room temperature.
5. Add 15 μL of SDS sample buffer (yielding a final vol of 60 μL), and mix.
6. Boil the sample for 2 min, and load an aliquot or the whole sample onto an SDS gel.
7. Perform SDS-PAGE, and transfer the proteins to nitrocellulose or PVDF membranes as described in vol. 1 of this series (*see* Notes 3 and 4).
8. Stain the proteins on nitrocellulose or PVDF membranes with Ponceau S to check for transfer efficiency. Incubate the membrane for 5 min in a solution of 0.2% Ponceau S in 3% acetic acid; rinse the membrane with H_2O until the protein bands are visible: The membrane can be photographed or pencil marked at this stage for documentation of the protein pattern. The Ponceau S staining disappears again during the following incubation steps.
9. Incubate the membrane for at least 30 min with approx 20 mL of blocking solution to prevent nonspecific binding. If necessary, the glycoprotein detection process can be interrupted at this stage and the membrane kept for a longer period (e.g., overnight) at 4°C.
10. Wash three times for 5 min with approx 50 mL of TBS each.
11. Incubate the membrane with 10 μL of antidigoxigenin-AP (750 U/mL) in 10 mL of TBS for 60 min at room temperature.
12. Wash three times for 5 min with approx 50 mL of TBS.
13. Incubate the membrane with 37.5 μL of 5-bromo-4-chloro-3-indolyl-phosphate (50 mg/mL in dimethylformamide) and 50 μL of 4-nitroblue tetrazolium chloride (75 mg/mL in 70% dimethylformamide) in 10 mL of buffer 2. This staining solution has to be prepared fresh. The reaction is normally complete within a few minutes, but can be extended overnight, e.g., if very little protein is present (*see* Note 5). Glycoproteins appear as gray to black bands on an almost white background. The reaction is stopped by rinsing the membrane with H_2O. The color does not fade, and the blots can be dried and stored for documentation (*see* Note 6).

Procedure 2: Oxidation and Labeling on Membranes (*see* Note 7)

1. Separate the proteins by SDS-PAGE, and transfer to nitrocellulose or PVDF membranes (*see* Note 8).
2. Wash the membrane three times for 5 min with approx 50 mL PBS. Do not use TBS since Tris interferes with the subsequent digoxigenin labeling.

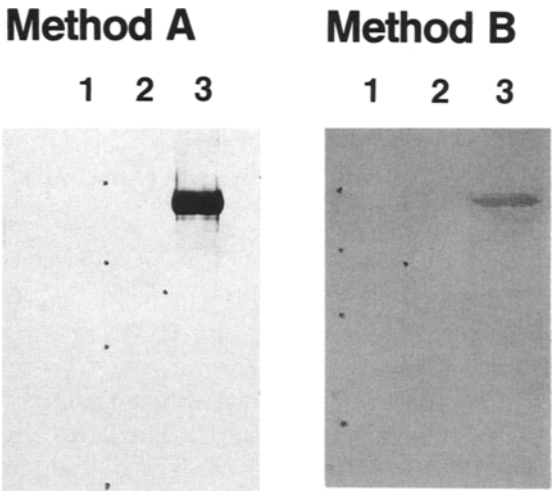

Fig. 2. The following proteins (1 μg) were loaded on an SDS gel and transferred to nitrocellulose. Lane 1, mol-wt standards (96, 55, 36, and 21 kDa); lane 2, creatinase (negative control protein); lane 3, transferrin; for procedure 1, the proteins were oxidized and digoxigenylated in solution, and for procedure 2, on the membrance as described, respectively. The little dots correspond to the position of the protein bands after Ponceau S staining.

3. Incubate the membrane with 10 m*M* sodium metaperiodate in 10 mL buffer 1 for 20 min at room temperature.
4. Wash three times for 5 min with approx 50 mL PBS.
5. Incubate the membrane with 1 μ*M* digoxigenin-succinyl-ε-amino caproic acid hydrazide (1 μL of a 5 m*M* solution in dimethylformamide) in 5 mL buffer 1, for 60 min at room temperature.
6. Wash three times for 5 min with approx 50 mL of TBS, and proceed further according to procedure 1 (from step 8).

Figure 2 illustrates these two procedures for the general detection of the glycoprotein transferrin (*see* Note 9).

Selective Detection of Sialic Acid Containing Glycoproteins

Since sialic acids are more susceptible to periodate oxidation than other sugars, the labeling procedure can be made specific for sialic acids by selecting the appropriate oxidation conditions. When using 1 m*M* periodate at 0°C for 20 min, only sialic acids are oxidized and subsequently labeled (*see* Note 10). This can be done either in solution or with the already transferred proteins. After the oxidation step, the protocols are exactly as described earlier.

PROCEDURE 1

Use a final concentration of 1 m*M* sodium metaperiodate in buffer in step 2, and incubate for 20 min at 0°C (add, e.g., 10 μL 3 m*M* periodate

solution). In step 3, the final concentration of sodium bisulfite solution should also be 1 m*M* (add 10 μL of a 4m*M* bisulfite solution).

PROCEDURE 2

In step 2, treat the membrane with 1 m*M* sodium metaperiodate in buffer 1 for 20 min at 0°C. All other steps are identical to the general procedure 2.

Selective Detection of Terminal Galactose Containing Glycoproteins

The enzyme galactose oxidase can be used to create specifically aldehyde groups at the C-6 position of terminal galactose residues of glycan chains. This allows the selective labeling of these groups with DIG-hydrazide. This particular procedure works much better on already blotted glycoproteins compared to doing it in solution. The digoxigenylation is carried out simultaneously with the oxidation reaction.

1. Incubate the membrane (dot or Western blot) for 30 min at room temperature with 20 mL of TBS, containing 1% BSA, and then wash for 5 min with 50 mL of buffer 4.
2. Incubate the membrane for 15 h at 37°C with 7.5 U galactose oxidase and 2 μL of digoxigenin-succinyl-ε-amino-caproic acid hydrazide solution (5 m*M* in dimethylformamide) dissolved in 10 mL of buffer 4.
3. Wash the membrane twice for 5 min with approx 50 mL TBS, and proceed with the blocking and digoxigenin detection as described earlier.

Figure 3 displays an example of using galactose oxidase for detecting terminal galactose containing glycoproteins in the range of 10–100 ng (*see* Note 11).

Structural Analysis of Glycoprotein Carbohydrate Chains Using Digoxigenin-Labeled Lectins

The suitability of employing the well-known carbohydrate binding specificities of lectins for the structural analysis of glycoproteins is well established (*see* refs. in [2]). It was logical therefore to use digoxigenylated lectins for the structural analysis of glycoproteins on membranes, thus combining the structural specificities of the lectins with a very sensitive detection system.

Since the conclusions on the carbohydrate structures present are entirely dependent on the type of lectin used, that is, in essence on the knowledge about its binding specificity, the lectins that are used for this purpose have to be chosen carefully. Examples of suitable lectins are listed in ref. (2), together with their respective carbohydrate binding specificities (*see* Materials, item 22 and Note 12).

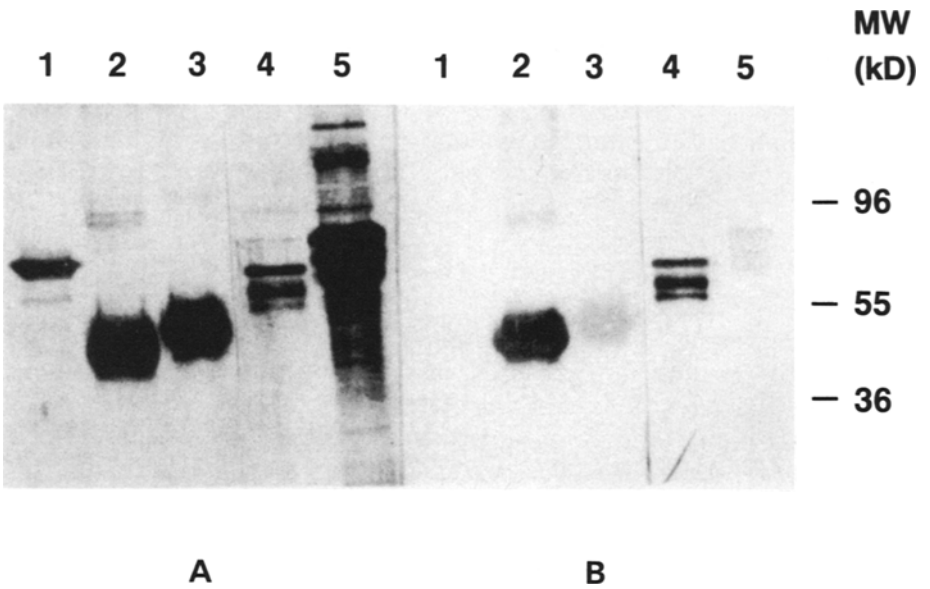

Fig. 3. The following glycoproteins (2 μg) were loaded on SDS gels and transferred to nitrocellulose. Lane 1, CpY; lane 2, α_1-acid glycoprotein, digested with neuraminidase; lane 3, α_1-acid glycoprotein; lane 4, fetuin, digested with neuraminidase; lane 5, fetuin. Blot A was oxidized chemically according to the standard procedure and blot B with galactose oxidase as described in Methods.

Glycoproteins to be analyzed with DIG-lectins are transferred to a membrane and incubated with the lectin of interest according to the following protocol:

1. Protein staining with Ponceau S, as described earlier, can be performed optionally prior to the lectin incubation.
2. Incubate the membrane for at least 30 min with approx 20 mL blocking solution (*see* Note 5).
3. Wash twice for 5 min with approx 50 mL TBS and once for 5 min with buffer 3.
4. Add the required amount of DIG-lectin solution to 10 mL buffer 3, and incubate the membrane for 60 min in this solution; the amounts required depend on the individual DIG-lectins. The DIG-lectins listed in Materials, item 22 are used as follows: AAA, 1 μg/mL; DSA, 1 μg/mL; GNA, 1 μg/mL; SNA 1 μg/mL; MAA, 5 μg/mL; PNA, 10 μg/mL; Con A, 20 μg/mL;RCA-120, 10 μg/mL; PHA-L, 5 μg/mL; WGA, 10 μg/mL. For other DIG-lectins, look for the recommended concentration in the respective pack inserts.
5. Wash three times for 5 min with approx 50 mL TBS.
6. Incubate the membrane with 10 μL of antidigoxigenin-AP conjugate (750 U/mL) in 10 mL TBS for 60 min.

7. Wash three times for 5 min with approx 50 mL TBS.
8. Perform the staining reaction with BCIP and NBT as described earlier.

Figure 4 shows an example where a set of glycoproteins is incubated with five different DIG-lectins (*see* Note 13).

NOTES

1. The presence of detergents (e.g., Triton X-100, Nonidet P-40, SDS) up to a concentration of 0.25% during oxidation and labeling does not cause interferences, with the exception of sugar containing detergents like octylglucoside. 2-Mercaptoethanol, dithiothreitol (>0.1%), glycerol (>0.01%), and amino groups containing buffers, such as Tris and glycine, have to be avoided when using procedure 1. It should also be taken into account that such substances could be present as stabilizers in enzyme preparations that might be used, e.g., for deglycosylation of the sample.

   The detection limit varies for each glycoprotein. After a 60-min incubation in the staining solution, the following amounts of glycoproteins are detected (using dot blots):
   a. α1-Acid glycoprotein: 1 ng;
   b. Fetuin: 1 ng;
   c. Transferrin: 5 ng;
   d. Carboxypeptidase Y: 10 ng.
2. It has been determined that 5 m*M* sodium metaperiodate and 5 m*M* sodium bisulfite as final concentrations in the oxidation solution result in the highest specificity of the overall labeling reaction. Oxidation with periodate has to be performed in the dark in order to prevent side reactions.
3. Glycoproteins, oxidized and labeled in solution, can also be applied directly onto membranes (dot blots); in this case, do not use detergent to denature the protein sample, since it will interfere with protein binding to the membrane. The use of nitrocellulose membranes for this purpose is also recommended, since PVDF membranes have to be prewetted before use, which will cause diffusion of the protein spots.
4. Because of the higher protein binding capacity, PVDF-type membranes are very well suited for this type of analysis, especially for detecting low amounts of glycoproteins. Nylon membranes are not recommended with this procedure, since they generate too much background.

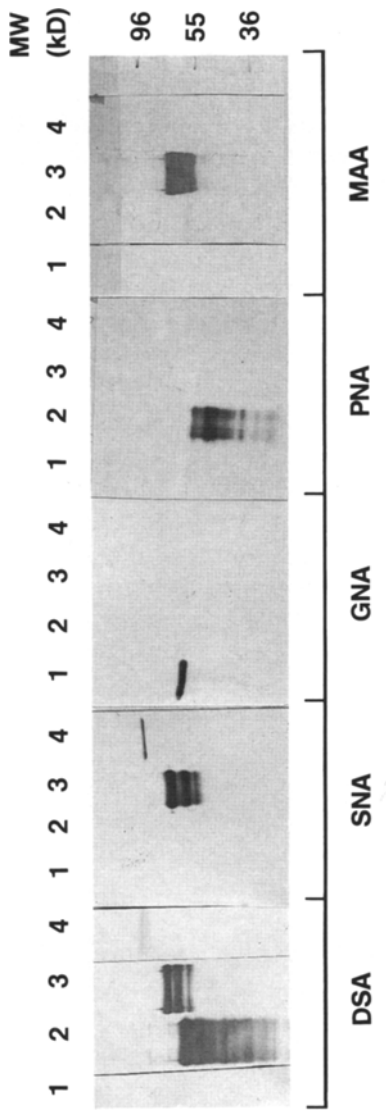

Fig. 4. The four glycoproteins (2 μg) were applied to SDS-PAGE and transferred to nitrocellulose. Five blots containing the same set of four glycoproteins were then treated individually with one of the five digoxygenylated lectins as described in Methods.

5. To reduce background on prolonged incubation with the staining solution when analyzing small amounts of glycoproteins, the membrane should be kept in the blocking solution (step 1) for longer than 30 min (e.g., overnight).
6. The alkaline phosphatase detection system with BCIP and NBT as substrates worked in our hands very satisfactorily and is one of the most sensitive visible detection systems available so far for the analysis of membranes. However, other anti-DIG conjugates (e.g., anti-DIG-peroxidase [POD], anti-DIG labeled with fluorescence markers) can also be used and are commercially available, if these systems are preferred for some reason. When using anti-DIG-POD, for instance, the same protocols as described above for anti-DIG-AP can be applied, and various substrates for the peroxidase reaction producing all sorts of different colors are available. There are also chemoluminescent substrates available, both for AP and POD, which can be used and are even more sensitive for certain applications than the colored products.
7. The advantages of procedure 2 are (a) components in the glycoprotein solution that could prevent labeling in solution or lead to unspecific labeling of nonglycoproteins are removed by performing the SDS-PAGE prior to the oxidation and labeling steps, and (b) the mobility of the proteins in SDS-PAGE is not altered, thus retaining the high resolution power of the SDS-PAGE. (The digoxigenylation of glycoproteins prior to electrophoresis causes a broadening of the protein bands.)

   Nonglycosylated proteins can appear as white bands or spots on a slight pink to gray background with this procedure. This is caused by a certain degree of digoxigenylation of the membrane, which does not take place at protein covered areas. When using method 2, sialic acids containing glycoproteins (e.g., fetuin) are generally more easily detectable than those without sialic acids.
8. Procedure 2 can be used with transfer and dot blots; when using dot blots, the first washing step can be omitted. After blotting with Tris-glycine buffers, the membrane has to be washed carefully to remove Tris and glycine, which interfere with the digoxigenylation step.

   In general, the sensitivity of procedure 2 is lower as compared to procedure 1. This holds true especially for glycoproteins not containing sialic acids. Approximate detection limits for the following glycoproteins on dot blots are:
   a. α_1-Acid glycoprotein: 5 ng;
   b. Fetuin: 5 ng;
   c. Transferrin: 25 ng;
   d. Carboxypeptidase Y: 50 ng.

9. Positive and negative controls should always be included in all of these experiments. Purified *E. coli* proteins (e.g., recombinant creatinase) are suitable as negative control, as are glycoproteins, such as carboxypeptidase Y or transferrin, after they have been completely deglycosylated with *N*-glycosidase F. Be aware of the presence of nonenzymatically linked glucose or fructose on many serum proteins (e.g., BSA), which will react positive especially when using Procedure 1.
10. As positive and negative controls for the sialic acid specific detection procedure, fetuin and asialofetuin can be used. Only fetuin will react under these conditions.
11. Sialic acid containing glycoproteins, such as fetuin and α_1-acid glycoprotein, can be rendered sensitive to galactose oxidase by incubation with neuraminidase (e.g., from *Arthrobacter ureafaciens*); for this purpose, neuraminidase (e.g., 0.1 U/mL) should be included in step 2 of Selective Detection of Terminal Galactose Containing Glycoproteins.
12. Positive and negative control proteins should always be included in the analysis to verify the carbohydrate specifities of the individual lectins. The lectins used for this type of carbohydrate analysis are tested primarily with glycoproteins from yeast and animal sources; when analyzing glycoproteins from plants or bacteria, different carbohydrate structures may be recognized also by the various lectins.
13. The sensitivity depends greatly on the respective glycoprotein and also varies somewhat between the individual lectins. Generally, the detection limit ranges between 1 and 10 ng glycoprotein for dot blot samples.

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