Rapid Determination of Glycoproteins and Glycopeptides by Periodic Acid Schiff Reagent Dot-Blotting Assay on Nitrocellulose Membrane

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We present here an improved practical approach of determination of glycoproteins and glycopeptides by directly dot-blotting samples on nitrocellulose membrane and staining the membrane with periodic acid Schiff (PAS) reagent. We demonstrate that it is possible to eliminate the time-consuming fixation step as in the staining of glycoproteins in polyacrylamide gel after electrophoresis and to shorten other staining steps. At least 0.1 μg of glycan can be detected within 40 min. Poly(vinylidene fluoride) membrane is an alternate choice for PAS staining. The choice of a proper membrane with smaller pore is important for the determination of glycopeptides. For the determination of glycopeptides of 3000–5000 molecular weight, a membrane with 0.1-μm pores is the best suited. As various detergents and buffers do not prevent the binding of proteins onto membrane, this technique could be used effectively for screening glycoproteins and glycopeptides.

INTRODUCTION

Peroxidases (EC. 1.11.1.7) are ubiquitous enzymes throughout the plant kingdom and play important roles in plant growth and development, such as auxin catabolism, ethylene biosynthesis, lignin formation, and defense against pathogens (Gaspar et al., 1982). Peanut peroxidases have been intensively investigated (van Huystee and Chibbar, 1987; van Huystee, 1990). There are mainly two isozymes, namely cationic and anionic peroxidases, released in suspension cell culture medium. Both of them contain around 20% carbohydrate (Hu and van Huystee, 1989). Probably, glycosylation is the most important posttranslational modification. In recent years, research interest in glycosylation of glycoproteins has intensified because continued investigation of glycoproteins has indicated that the carbohydrate moiety of glycoproteins confers important biological functions, such as protease resistance, antigenicity, protein targeting, antibody–antigen binding, and cell–cell interactions (Olden et al., 1982; Paulson, 1989; Takegawa et al., 1989; Wright et al., 1991; Kalisz et al., 1991; Hu and van Huystee, 1989). A sensitive and rapid determination of glycans is desired for the purification and identification of glycoproteins and glycopeptides. In the past few years, several dot-blotting assay methods to detect glycoproteins have been reported (Faye and Chrispeels, 1985; Weitzhandler and Hardy, 1990; Hsi et al., 1991; Buee et al., 1991). These methods use mainly lectins to probe glycans and, in some cases, are selective because of the sugar binding specificity of lectin. When we study the structure and function of glycans of peanut peroxidases, we distinguish glycopeptides by directly dot-blotting samples onto nitrocellulose (NC) membrane and staining the membrane with periodic acid Schiff (PAS) reagent using an improved staining method. We demonstrate here that it is possible to eliminate a time-consuming fixation step and to shorten staining and washing steps.

MATERIALS AND METHODS

Reagents. NC membranes (BA 85, 0.45 μm; PH 79, 0.1 μm) were purchased from Schleicher and Schuell (Keene, NH). Bio-Gel P6 (200–400 mesh) was purchased from Bio-Rad Laboratories,

L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK)–trypsin from Worthington Biochemical Corp. (Freehold, NJ), fetuin from Sigma Chemical Co., and basic fuchsin from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Cell Culture and Peroxidase Purification. Peanut cells (*Arachis hypogaea L.* var. Virginia 56R), derived from cotyledon slices, were routinely cultured for 14 days (Hu et al., 1987). The cationic peroxidase from the spent growth medium was purified as described (Sesto and van Huystee, 1989).

Tryptic Digestion of Peroxidase. After removal of the heme moiety by acetic acetone (Chibbar et al., 1984), peroxidase was incubated with TPCK–trypsin at 37 °C for 40 min. The ratio of substrate:TPCK–trypsin was 30:1 (w/w). The trypsin was inactivated by boiling for 3 min.

Gel Filtration Chromatography. The TPCK–trypsin-digested peroxidase was applied to the Bio-Gel P6 column (1.5 cm × 90 cm). The column was eluted with 0.1 M ammonium acetate, pH 7, containing 0.02% NaN₃ at room temperature at a flow rate of 8 mL/h. Fractions of 2 mL were collected. Glycopeptide fractions were identified by dot-blotting assay on NC membrane or phenol–sulfuric acid method.

Schiff Reagent Preparation. The Schiff reagent was prepared according to the method of Segrest and Jackson (1972) with slight modifications. Five grams of basic fuchsin was dissolved in 1 L of boiling distilled water. After cooling, 100 mL of 1 N HCl and 5 g of sodium metabisulfite were added. The solution was placed in the dark overnight. After 10 g of activated charcoal was added, the solution was twice vacuum-filtered through four layers of Whatman No. 1 filter paper. The filtrate was clear and colorless.

PAS Staining of Glycoproteins or Glycopeptides on NC Membrane. The procedure is based on modified methods (Segrest and Jackson, 1972; Konat and Mellah, 1984). NC membrane was wetted with distilled water, followed by removal of excess water with filter paper. Then, 5 μL of sample solution was dot-blotted on the membrane using a Justor 1100 DG digital pipet (Nichiryo Co. Ltd.). After air-drying, the membrane was soaked in turns in 0.2% aqueous periodic acid, 0.2% sodium metabisulfite, Schiff reagent, and 0.2% sodium metabisulfite in 40% ethanol and 5% acetic acid for 10 min, or varying time as indicated for each, at room temperature and then washed with one change of 10% acetic acid.

Phenol–Sulfuric Acid Color Reaction. Sugar detection was done according to a modified method (Dubois et al., 1956). Briefly, 0.25 mL of sample was mixed with 0.3 mL of 5% phenol, and then 2 mL of concentrated sulfuric acid was added quickly. The absorbance was measured at 490 nm on Shimadzu UV-160 spectrophotometer after 30 min.

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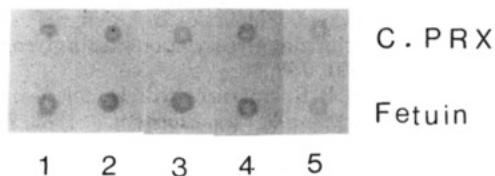
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Figure 1. Comparison of various staining times of dot-blotted glycoproteins on NC membrane with PAS. Five microliters of sample (0.4 µg of glycoprotein/µL) was dot-blotted on a BA 85 NC membrane. (Upper row) cationic peroxidase (C. PRX); (lower row) fetuin. The staining time of each step was (lane 1) 5 min; (lane 2) 10 min; (lane 3) 20 min; (lane 4) 40 min; and (lane 5) 20 min, but without the last treatment of sodium metabisulfite.
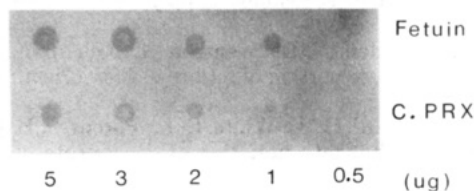

Figure 2. Sensitivity of PAS staining of dot-blotted glycoproteins on NC membrane. Five microliters of serially diluted samples containing 5, 3, 2, 1, or 0.5 µg of C. PRX or fetuin, respectively, was dot-blotted on a BA 85 NC membrane. (Upper row) fetuin; (lower row) C. PRX. Each staining step lasted 10 min.
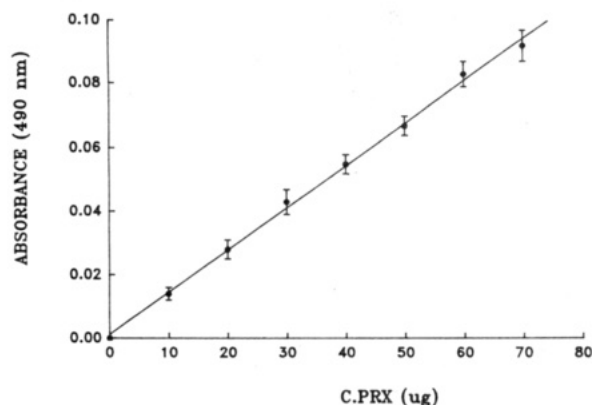

Figure 3. Sensitivity of phenol–sulfuric acid color reaction. A sample of 0.25 mL containing different amounts of C. PRX was mixed with 0.3 mL of 5% phenol. After addition of 2 mL of concentrated sulfuric acid, the absorbance was measured at 490 nm.

RESULTS AND DISCUSSION

PAS reagent can be employed to stain glycoproteins and other macromolecules with carbohydrate content and was successfully used in the staining of glycoproteins in polyacrylamide gels (Segrest and Jackson, 1972). As the gel impedes the contact of staining reagent with glycoproteins in the gel, a staining procedure takes more time and requires a protein fixation step before staining. NC membrane has a strong binding capacity with proteins, and staining chemicals can reach blotted glycoprotein easily. So, it is possible by protein blotting to eliminate the fixation step and greatly decrease the staining period. The reaction product of PAS and glycoproteins or glycopeptides on NC membrane presents magenta spots. To estimate the staining time and sensitivity of this method, two well-characterized glycoproteins were employed. Cationic peroxidase has a mass of 40 000 with 20% carbohydrate and fetuin has 6 glycans per molecule, which account for one-third of the whole molecular weight (Nilsson et al., 1979; Rupar and Cook, 1982). Figure 1 shows that there is no significant difference of staining intensity among 5, 10, 20, and 40 min. There is, however, a clear difference of staining intensity between treatments
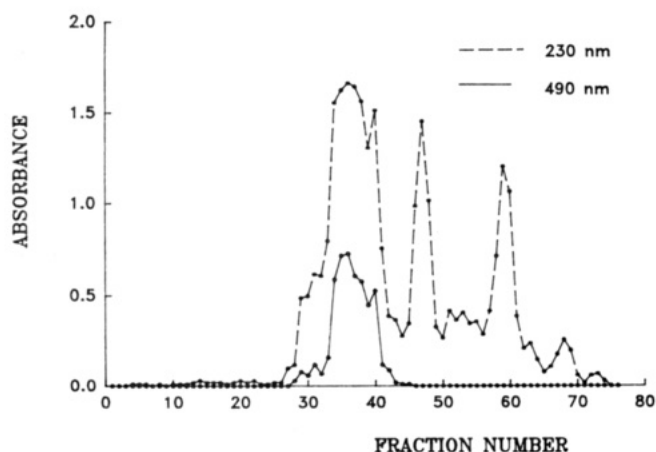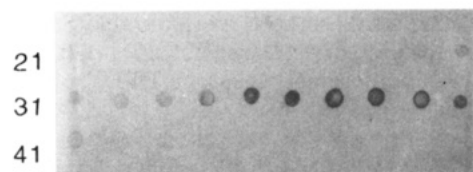

Figure 4. Detection of glycopeptide fractions of TPCK–tryptic cationic peroxidase eluted from Bio-Gel P6 column. Fifty milligrams of acetic acetone treated cationic peroxidase was incubated with TPCK–trypsin at 37 °C for 40 min. The column was eluted at 8 mL/h. Fractions of 2 mL were collected. (A) The absorbance at 230 nm was used to detect peptide fractions, and 0.25 mL of each fraction was used for the phenol–sulfuric acid method to identify glycopeptide fractions. (B) Five microliters of fractions 21–50 was dot-blotted on a PH 79 NC membrane. Each staining step of membrane lasted 10 min.

with and without sodium metabisulfite. Sodium metabisulfite appears to enhance staining. The additional treatment of a methanolic solution with sodium metabisulfite resulted in an increase of sensitivity (Konat and Mellah, 1984). Glycoprotein can be detected within at least 40 min. Figure 2 shows that 0.5 µg of cationic peroxidase, equal to 0.1 µg of carbohydrate, is the lower level, which may be detected. Because PAS reagent does not require any specific terminal sugars, it is suitable for staining a variety of glycoproteins. In contrast, 40 µg of cationic peroxidase, equal to 8 µg of carbohydrate, gave an absorbance of only 0.05 at 490 nm with the phenol–sulfuric acid method (Figure 3), another popularly used sugar-detecting technique. Compared with the phenol–sulfuric method, the dot-blotting assay of glycoprotein is more sensitive and less sample-consuming. Dot-blotting assays for glycopeptides are slightly more complex than those for glycoproteins. This is caused by the weaker binding of glycopeptides onto the membrane, especially when the glycopeptide has a shorter peptide and a large glycan. The second issue is that glycopeptides often have small mass, which allows glycopeptides to travel readily across the membrane with larger pores. So, it is important to select the proper membrane. The mass of TPCK–tryptic glycopeptides of cationic peanut peroxidase is around 3000–5000 on the basis of peptide lengths and masses of glycans, and the NC membrane with a 0.1-µm pore is the best suited membrane for the dot-blotting assay according to our experiences. Poly(vinylidene fluoride) (PVDF) membrane is another choice for PAS staining. Although nylon membrane has high binding capacity for proteins, it has high staining background with PAS reagent.

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As various detergents and buffer reagents, such as SDS, Tris, phosphate, and acetate, do not prevent the binding of proteins onto NC membrane (data not shown), the dot-blotting assay following chromatography or western blotting after electrophoresis is a useful tool to detect glycoprotein or glycopeptide fractions. Figure 4 shows the identification of glycopeptide fractions of TPCK-tryptic cationic peroxidase eluted from the P6 column. Both the PAS dot-blotting assay and the phenol–sulfuric acid method revealed that fractions 28–43 contained glycopeptides. The biological roles of glycosylation of peanut peroxidases can be studied in more details with this approach.

The procedure described here supplied a simple, rapid, and sensitive determination of glycoproteins and glyco-peptides.

ABBREVIATIONS USED

NC, nitrocellulose; PAS, periodic acid Schiff; C. PRX, cationic peanut peroxidase; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PVDF, poly(vinylidene fluoride).

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