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Fluorescent Staining of Glycoproteins on Polyvinylidene Difluoride Membrane with 8-Aminonaphthalene-1,3,6-Trisulfonate

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ABSTRACT

Here, we describe a simple and sensitive method that allows fluorescent detection of glycoproteins on polyvinylidene difluoride (PVDF) membrane. We used periodic acid oxidation of carbohydrate chains of glycoproteins and fluorescent labeling with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) by reductive amination. We developed an additional method to enhance the ability of PVDF to absorb glycoproteins by using non-glycoprotein lectin, such as wheat germ agglutinin (WGA), as a link between the PVDF membrane and glycoproteins, resulting in considerably increased detection sensitivity to glycoproteins.

INTRODUCTION

Protein glycosylation, a universal but complicated co- and post-translational modification of proteins in eukaryote cells (26), has been found to be very important to glycoprotein and many other biological processes (4,7,22,23,26). To comprehensively understand a sophisticated biological system, researchers often determine if a preparation contains glycoproteins or if a protein is glycosylated. For this reason, many staining methods have been developed to detect glycoproteins (1,2,5,8-14,17,18,20,21,24,27,28). These methods can be roughly classified into three general categories. One category is lectin blotting based on specific and noncovalent lectin binding to oligosaccharides of protein (11,12). Lectins are pre-biotinylated or avidin conjugated with horseradish peroxidase or alkaline phosphatase (13,17,20,27). The resulting complexes are then revealed either by a chemiluminescent or a colorimetric reaction. Alternatively, lectins have

been labeled with fluorescent reagents (2,9,14). Lectin methods are usually expensive because they require large amounts of lectin. The next general category is periodic acid/Schiff (PAS) glycoprotein staining on polyacrylamide gel (28), nitrocellulose, or polyvinylidene difluoride (PVDF) membrane (24). Carbohydrates of glycoprotein are initially oxidized by periodic acid and subsequent staining with Schiff's reagent. The disadvantages of using PAS staining on gel are long incubations, multiple washings of the gel, and lower sensitivity, often resulting in microgram amounts of glycoproteins. PAS staining of glycoprotein electroblooded on nitrocellulose or PVDF membrane increases the sensitivity. However, because the Schiff-base reaction is reversible, the reaction cannot usually be completed, and some Schiff bases are unstable. The third category is binding hydrazide to periodate-oxidized glycoproteins in which hydrazide has been derivatized with enzymes (10), biotin (21), avidin/streptavidin (1), and fluorescent dyes (5,8,18).

Here, we describe an alternate method to detect glycoproteins on PVDF, which is simple, fast, sensitive, and economic. Glycoproteins on PVDF are sequentially subjected to periodic acid oxidation and stable fluorescent labeling with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) by reductive amination. We also describe a simple and novel method to enhance the ability of PVDF to absorb glycoproteins.

MATERIALS AND METHODS

Materials

ANTS was purchased from Molecular Probes (Eugene, OR, USA). Sodium cyanoborohydride (NaCNBH_3), calf fetuin, yeast invertase, bovine β casein, turkey ovalbumin (i.e., A7269), and wheat germ agglutinin (WGA) were all from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany), and PVDF membrane (ImmobilonTMP) and Milli-Q[®] water were from Millipore (Bedford, MA, USA). All other chemicals were of analytical reagent grade.

Dot-Blotting Proteins on PVDF

PVDF was wetted with 50% methanol/water for 2 min and rinsed briefly with water. After a brief air dry, PVDF was set onto a dot apparatus (Minifold[®]; Schleicher & Schuell, Keene, NH, USA). Then, protein sample in 0.1% TFA/water was dotted onto the membrane. To enhance the ability of PVDF to absorb glycoproteins, an appropriate amount of a non-glycosylated lectin in 0.1% TFA/water, such as WGA, was dotted onto the membrane 1 min before dotting a protein sample.

SDS-PAGE and Western Blotting

Protein sample solution was mixed with the equivalent volume of sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, 2% SDS, 0.01% bromophenol blue, and 30% glycerol, 1% 2-mercaptoethanol) and subjected to SDS-PAGE. SDS-PAGE was carried out in 12% gel on Mini-PROTEAN[®] II Cell. Electroblood was performed on a Trans-Blot[®] Cell in a transfer buffer (10 mM CHAPS, 10% methanol, pH 11.0, adjusted with Tris base) (both purchased from Bio-Rad Laboratories, Hercules, CA, USA). The electrophoretic voltage and current were 90 kV and 220 mA, respectively. The transfer was carried out for 1 h.

Fluorescent Detection of Glycoproteins on PVDF

Glycoproteins dot blotted or electroblooded on PVDF membrane were oxidized with 20 mL 1.5% periodic acid in 5% acetic acid/water for 20 min at room temperature in the dark. The membrane was washed with 40 mL water three times each for 2 min, soaked in 10 mL fluorescent-labeling solution (10 mg ANTS, 20 mg NaCNBH_3 in 50% DMSO, and 15% acetic acid in water) and incubated at 60°C for 45–60 min. After labeling, the membrane was washed with 40 mL water three times each for 2 min and allowed to air dry at room temperature. Glycoproteins were detected under a standard long-wavelength UV light, and photographs were taken to record the results.

RESULTS AND DISCUSSION

Jackson (16) was the first to introduce ANTS into fluorophore-assisted carbohydrate electrophoresis (FACE) to label oligosaccharides and monosaccharides by reductive amination. Complete labeling has been obtained with glucose, lactose, maltopentaose, and Gal-6-SO₃⁻ (15,16). Klockow et al. (19) reported that a faster ANTS derivatization of carbohydrates could be performed at 80°C for 2.5 h. However, our experiments showed the detection sensitivity of glycoproteins on PVDF decreased when the temperature was higher than 70°C. The reason might be that the ability of PVDF to absorb glycoproteins decreased with the increase in labeling temperature. We optimized the labeling conditions and found the suitable temperature and time were 60°C for approximately 45–60 min (data not shown). This gentler condition coincided with the fact that aldehydes (e.g., from the periodic acid-oxidized glycoproteins) react faster in reductive amination than carbohydrates because the latter are either in pyranose or furanose forms.

Figure 1 shows the results of fluorescent detection of glycoproteins dot blotted on PVDF. Typically, 1.2 µg of four glycoproteins—calf fetuin, inver-

tase, bovine βcasein, and turkey ovalbumin—are visualized with strong fluorescence under UV (Figure 1, lane A). The oligosaccharides of the first three glycoproteins belong to N-linked complex (6), N-linked high mannose (25), and O-linked carbohydrates (<http://www.ncbi.nlm.nih.gov>), respectively. Turkey ovalbumin A7269 might contain N-linked high mannose or hybrid oligosaccharides or both of the two types. This indicates that our method can detect glycoproteins containing the common structure types of oligosaccharides. As expected, WGA, a non-glycoprotein, was negatively detected even when 16 µg WGA were dot blotted onto PVDF. Two other non-glycoproteins, trichosanin and recombinant arginyl-tRNA synthetase expressed in *E. coli*, were also tested and negatively detected by this method (data not shown), indicating that the specificity for glycoproteins is high. The sensitivity mainly depends on the amount of carbohydrate on glycoproteins and the structure type of carbohydrate.

Because of impediments resulting from a highly hydrophilic oligosaccharide moiety of glycoproteins, the hydrophobic PVDF membrane binds to glycoproteins more weakly than to non-glycoproteins. Therefore, we designed a very simple strategy to enhance the ability of PVDF to absorb glycoproteins. We dot a non-glycosylated lectin, such as WGA, Concanavalin A (Con A), or peanut agglutinin (PNA) onto

PVDF, then dot glycoprotein on the spot of the lectin. Glycoproteins are fixed onto PVDF through the action between the glycoprotein and lectin. Because no covalent saccharides are attached to this kind of lectin, the lectin does not interfere with the detection of glycoprotein. Figure 1, lane B, shows that 200 ng glycoproteins dotted onto PVDF could not be detected using the standard method. However, using the technique to enhance the dot blot by adding WGA, 50 ng calf fetuin and invertase were clearly detected as glycoproteins, and the fluorescent intensity for the turkey ovalbumin A7269 increased to some extent (Figure 1, lane C). The carbohydrate specificity of WGA can account for the difference in the increments of fluorescent intensity of these glycoproteins. WGA binds specifically to GlcNAc and NeuAc. The clustering sialyl residues may be necessary for the strong interaction of sialoglycoproteins with WGA (3). Fetuin contains many NeuAc and GlcNAc residues (6), and invertase contains many GlcNAc residues (25). Therefore, by adding WGA, the fluorescence intensity of fetuin or invertase increased more significantly than that of A7290 or βcasein, which contain less GlcNAc and no NeuAc residues. This indicates that one limitation of the method for enhancing the dot blot by adding a lectin (e.g., WGA) is that it is only effective with the lectin-binding glycoproteins. However, this method could provide some information about the composition and structure of oligosaccharides on glycoproteins. To broaden the lectin reactivity, a lectin mixture of WGA, Con A, and PNA can be used.

The combination of SDS-PAGE and electroblotting onto PVDF membrane has been an excellent tool for the separation and micropreparation of proteins. Because it is very stable to strong acids or bases even at 100°C, PVDF membrane carrying the purified protein can be directly subjected to N- or C-terminal microprotein sequence analysis, hydrolysis for the analysis of amino acid composition, or monosaccharide composition of glycoprotein. Here, we extend the periodic acid/ANTS-reductive amination method to fluorescent detection of glycoproteins on PVDF electroblotted after SDS-PAGE. Figure 2 shows the

Figure 1. Fluorescent detection of glycoproteins dot blotted onto a PVDF membrane by periodic acid/ANTS-reductive amination. 1, WGA; 2, fetuin; 3, invertase; 4, βcasein; and 5, turkey ovalbumin (A7269). The amount of each protein was: lane A, 1.2 µg without WGA; lane B, 200 ng without WGA; and lane C, 50 ng with 2 µg WGA.

Figure 2. Fluorescent detection of glycoproteins electroblotted onto a PVDF membrane by periodic acid/ANTS-reductive amination. Ten micrograms of each protein were subjected to SDS-PAGE and then electroblotted onto a PVDF membrane. Lane A, turkey ovalbumin (A7269); lane B, βcasein; lane C, invertase; lane D, fetuin; and lane E, WGA.

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fluorescent staining pattern of 10 μ g proteins electroblotted on PVDF. Fetuin, invertase, β casein, and turkey ovalbumin A7269 showed obvious fluorescence, whereas WGA was not visualized under UV, as expected, even when 30 μ g WGA were loaded onto the gel.

This report primarily demonstrates that periodic acid/ANTS-reductive amination fluorescent detection of glycoproteins on PVDF is a simple, fast, and sensitive method. It is also an economic approach compared to the other methods, such as lectin blotting, avidin/streptavidin, biotin, and other fluorescent reagent methods because ANTS is less expensive but more stable. ANTS can be labeled stably on glycoproteins by reductive amination. (There was almost no decrease in the fluorescent intensity of the labeled glycoprotein on PVDF in one week). Another advantage of this method is that after fluorescent labeling, very low background is observed on PVDF under UV, and no background is observed under visible light. This is because three negatively charged ANTS do not easily bind to PVDF, but if bound, it is easy to wash ANTS from PVDF. Therefore, the same membrane can be re-stained using other dye (e.g., Coomassie®Brilliant Blue) to visualize all proteins, allowing discrimination between glycoproteins and non-glycoproteins. Our data (unpublished) showed that the method described above could be applied to the detection of glycoproteins that are separated by 2-D gel electrophoresis and electroblotted onto PVDF.

This method could be improved by studying the conditions for the optimal-binding activity of one lectin or lectin mixture on PVDF membrane or by the use of a fluorescence imager, but further work is necessary.

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