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A Novel Method for Detection of Glycoproteins on Sodium Dodecyl Sulphate Polyacrylamide Gel Using Radio-Iodinated Tyrosine

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Abstract: The aim of this study is to develop a novel method for detection of glycoproteins on polyacrylamide gel. In this method, radio-iodinated-tyrosine (^{125}I-tyrosine) was conjugated to glycoprotein by schiff's base mechanism on the sodium dodecyl sulfate-polyacrylamide gel. Ovalbumin and Concanavalin A (Con A) were used as a glycosylated and a non-glycosylated model proteins, respectively. The proteins were separated in SDS-PAGE and oligosaccharides on the glycoprotein were oxidised using periodic acid to produce aldehydes than ^{125}I-tyroine was conjugated to aldehyde groups without using reducing agent like Sodium Metabisulfite. The radio-iodinated glycoprotein on gel was scanned using a Multi-Photon Detection (MPD) scanner. The electrophoretic analysis of ovalbumin and Con A were performed and stained with Coomassie brilliant blue to identify total proteins, while MPD detection of glycoproteins using ^{125}I-tyrosine selectively detected ovalbumin. Present results showed that MPD enhanced glycoprotein detection method can be used as a sensitive tool for the detection of glycoproteins on polyacrylamide gel.

Key words: Glycoproteins, multi-photon detection, radio-iodinated-tyrosine

INTRODUCTION

As a part of the systems biology efforts (Auffray *et al*., 2003; Ideker, 2004; Kell and Oliver, 2004; Kitano, 2002; Stevens, 2004; Van der Greef *et al*., 2004) aiming at a nearly complete understanding of the complexity of living organisms, glycomics and glycoproteomics deserve particular attention. This is due to the fact that many important processes in mammalian cells are mediated through various actions of glycoproteins and proteoglycans (Varki *et al*., 1999).

Post-Translational Modification (PTM) is an important feature of a proteome, frequently conveying a specific biological activity or role to a protein. Modifications can occur at a single or at multiple sites, often in varying forms (Krishna and Wold, 1998). Protein glycosylation has long been recognised as a very common post-transactional modification. Typically, carbohydrates are linked to serine or threonine residues (O-linked glycosylation) or to asparagines residues (N-linked glycosylation) (Varki *et al*., 1999).

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High-sensitivity glycoprotein analyses are of particular interest in modern biomedical and clinical research (Novotny and Mechref, 2005). The improved analytical methodologies in glycoproteomics are clearly a key to the future of glycobiology research. The importance of glycosylation in health and sickness has been increasingly evident, for nearly all major human diseases have their glycoprotein attributes represented in the constantly growing number of biomarkers. This includes the most serious illnesses, such as various types of cancer, cardiovascular disease and immunological disorders (Dennis *et al*., 1999; Lowe and Marth, 2003). Additionally, a detailed knowledge of glycosylated structures could aid research efforts in developmental biology, plant sciences, agricultural research, etc.

Methods were developed to stain carbohydrates on proteins separated by SDS-PAGE, Glycoproteins are detected on the SDS-PAGE and after blotting on PVDF or nitrocellulose membrane using lectins (Linton *et al*., 2002), Periodic acid-schiff stain (Doerner and White, 1990), enzyme hydrazides (Gershoni *et al*., 1985; Keren *et al*., 1986), fluorescent hydrazide (Steinberg *et al*., 2001), digoxigenin hydrazide (Bouchez-Mahiout *et al*., 1999) and enzyme base avidine-biotin technology (Bayer *et al*., 1987).

General detection methods for glycoproteins are based on the periodate oxidation of carbohydrates resulting aldehyde groups on the protein to reacts with primary amine containing molecules to form schiff bases (Doerner and White, 1990) or to react with hydrazides to form hydrazones (Steinberg *et al*., 2001; Bouchez-Mahiout *et al*., 1999; Bayer *et al*., 1987). The oxidation and coupling reaction can be performed in solution (Haselbeck and Hösel, 1990), on blot (Steinberg *et al*., 2001; Bouchez-Mahiout *et al*., 1999; Bayer *et al*., 1987; Haselbeck and Hösel, 1990) or on gel (Doerner and White, 1990; Steinberg *et al*., 2001).

The aim of the present study was to develop a convenient and sensitive method for the detection of glycoproteins on gel. Based on the schiff base reaction in the absence of reducing agent, we developed a novel method for of radiolabelling and detection of glycoproteins using ^{125}I-tyrosine after periodic oxidation (Fig. 1).

The quantification of radiolabels by scintillation counting has long been one of the most reliable methods for accurate, quantitative measurement in biochemical experiments. This method has been employed in the context of proteomics (Gygi and Aebersold, 1999; Gygi *et al*., 2000) and does offer gain in an absolute sensitivity and dynamic range.

The common alternative, autoradiography, suffers from the problems that high-sensitivity requires long measurement times, which are incompatible with high-throughput and only a limited dynamic range (about 10^2) is achievable with film detection. Phosphor imaging provides some improvement, but even this technique is not really adequate for functional proteomics. A substantial improvement in the detection and quantification of radiolabels has recently been achieved with the

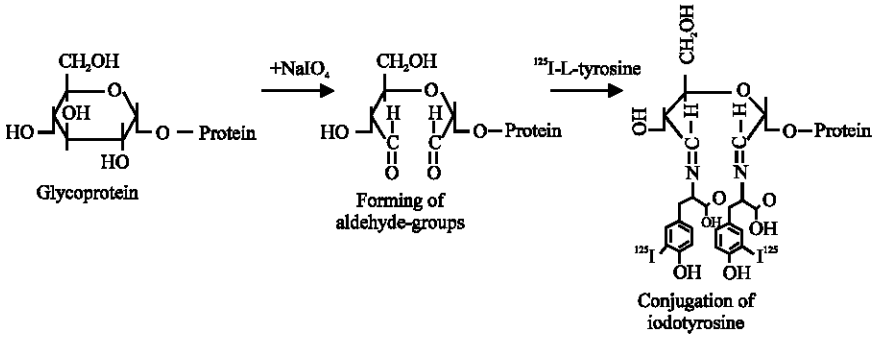

Fig. 1: Diagrammatic representation of radioiodination of glycoproteins using ^{125}I-L-tyrosine

development of Multi-Photon Detection (MPD) methods based on the coincident single particle detectors for quantification of multiple particles/photons in a single radioactive decay process (Kleiner *et al*., 2005).

MATERIALS AND METHODS

Materials

L-tyrosine, hen egg white albumin (Ovalbumin) and Coomassie Brilliant Blue R 250 were from Fluka Denmark. Novex® Tris-Glycine Pre-Cast gels were obtained from Invitrogen, Denmark. Concanavalin A *Canavalia ensiformis* (Jack Bean) Type III, Periodic acid and 1,4-Dithio-DL-threitol were from Sigma-aldrich, Denmark. 4X LDS sample buffer was from Kem-En-Tec, Denmark. Iodo-beads were from Pierce, IL, USA. Acetic acid glacial 100%, Glycerol 87%, Tween-20, Sodium acetate trihydrate was from Merck, CA, USA. Carrier free Sodium ^{125}I-Iodide was from Hartmann analytic, Germany. All the buffer and reagents throughout the experiments prepared using Milli Q water.

Radio-Iodination of L-Tyrosine

L-tyrosine was radiolabelled using Iodo-bead method. Two Iodo-beads were washed with 1 mL of 0.1 M sodium phosphate buffer, pH 6.0. Dried on filter paper and placed in a reaction vial containing 0.5 mL of 0.1 M sodium phosphate buffer, pH 6.0. Ten microliters of 1 mCi carrier free Na-iodide^{125} was added to the reaction vial and reaction was allowed for 5 min at room temperature. Forty microliters of 0.5 mg mL^{-1} of L-tyrosine was added to the reaction vial and incubated for 30 min. Iodinated L-Tyrosine was collected into a vial containing 40 μL of 0.5 mg mL^{-1} L-Tyrosine to stop the reaction and stored at 4°C.

Coomassie Staining of Total Proteins

SDS-PAGE was performed using 12.5% Tris-glycine pre-cast gels. Protein samples (1 μL of 10 μg μL^{-1} ovalbumin or Con A) were added to 9 μL of sample buffer and placed in boiling water bath for 3 min. The protein samples and molecular weight marker were applied to then electrophoresis was performed for 2 h at 120 V using Bio-Rad model 160/1.6 power supply. The gel was stained overnight with 0.1% Coomassie brilliant blue in 45% ethanol and 10% acetic acid then washed with 40% ethanol and 10% acetic acid, followed by incubation in 5% glycerol.

Detection of Glycoprotein

SDS-PAGE was performed using 12.5% Tris-glycine pre-cast gel. Protein samples (1 μL of 10 μg μL^{-1} ovalbumin or Con A) were added to 9 μL of sample buffer and placed in boiling water bath for 3 min. The protein samples were applied on to gel and electrophoresis was performed for 2 h at 120 V using Bio-Rad model 160/1.6 power supply. The gel was incubated in 7.5% acetic acid for 30 min then transfer to oxidation solution containing 0.2% w/v periodic acid in 3% acetic acid and incubated for 1 h at RT in dark. The gel was washed 3 times for 5 min with Milli Q water then incubated in solution containing 1:10,000 ^{125}I-tyrosine in 3% acetic acid for 1 h at RT. The gel was washed 3 times for 5 min with 0.1M sodium acetate buffer, (pH 5.5) containing 0.05% Tween-20. Then transfer to solution with 40% ethanol, 5% glycerol for 5 min. Finally the gel was incubated in 5% glycerol for 30 min prior to dry in between sheets of cellophane for detection of glycoproteins using MPD scanner.

MPD Scanner

MPD imager has 2 identical detectors placed on each side of the gel. The gel was supported by a thin plastic film, which eliminates the gap between detectors and the gel. This results in high-

detection efficiency and a good spatial resolution. The detectors consist of a position sensitive photomultiplier coupled with a CsI(Na) scintillating pixel array. The pixel size is 1.8×1.8 mm with a 0.2 mm optical separation between pixels. The array itself consists of 20×20 = 400 pixels. Each detector determines both x and y coordinates for each event detected and then the event is assigned to a pixel defined by the event coordinates. After the user's preset acquisition time, the computerized gel support moves the gel to a new position by the same step along both x and y directions. Currently the period of the pixel array is 2 mm and usually 0.5 mm was chosen as measuring steps. The MPD images are acquired in two modes, coincident (C) and Non-Coincident (NC). In C mode background, radiation is excluded to better than 0.005 cpm per pixel. The NC mode has a ten-fold higher background, but its detection efficiency is three-fold higher. Typically ^{125}I-Iodide is detected in both C and NC modes. Software developed in-house is used for image generation, pattern recognition and quantitation.

RESULTS AND DISCUSSION

MPD is able to quantify radioisotopes which decay by the electron capture mechanism and hence emit multiple photons/particles essentially and simultaneously. Background radioactivity very rarely provides coincident emissions of defined energies and can therefore be rejected from the analysis. Coincidence detection of multiple emissions by MPD thus permits exquisitely sensitive detection for appropriate radiolabels well below background radiation levels. To demonstrate detection of glycoproteins by an MPD, we performed 1-D PAGE of the standard proteins ovalbumin and Con A. Oligosaccharides of the glycoproteins were oxidised to produce aldehyde groups (Fig. 1). Subsequently the SDS-PAGE gels are stained with ^{125}I-tyrosine to be compatible with gamma aquatint of multi-photon detection scanner. We also demonstrated that primary amines react even in acidic pH around 2-3. This pH will avoid oxidation of amino acids to form aldehyde groups due to low pH.

The electrophoretic analysis of ovalbumin and Con A (Fig. 2) stained with Coomassie brilliant blue showed both bands for ovalbumin and Con A while MPD detection of glycoproteins using ^{125}I-tyrosine showed one main peak for ovalbumin. Our results showed that MPD can be used as a sensitive method for detection of radio-iodinated glycoproteins on gels.

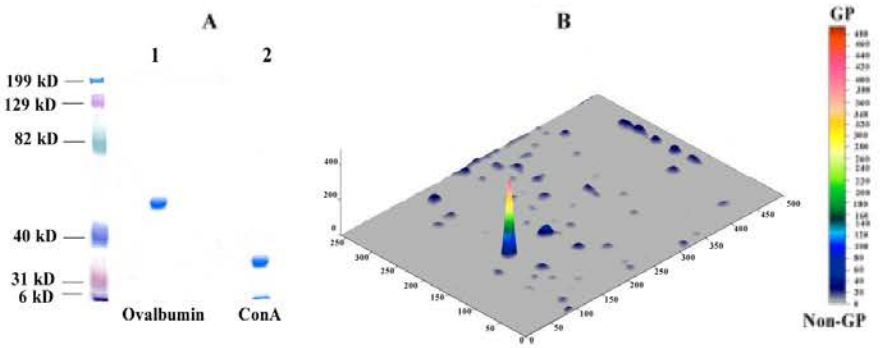

Fig. 2: Total protein and glycoprotein visualized using non-radioactive and radioactive methods. (A) Total protein was detected using Coomassie brilliant blue staining (lane 1: Ovalbumin; lane 2: Con A). (B) Glycoprotein was detected using radio iodinated L-Tyrosine. The multi-colour bar represents the intensity of detection (measured in cpm) and detection limit of glycoprotein (GP) verses non-glycoprotein (Non-GP) and background detection

The strategies in glycoprotein analysis may depend on the quantity of available sample. Microgram to milligram quantities of isolated complex glycoproteins, or a recombinant product, are now easily amenable to complete chemical characterization, although controlled cleavages by proteases or glycanases could still result in fairly complex mixtures of peptides, glycopeptides and glycans. Somewhat more challenging are the cases where glycoproteins have been chromatographically separated from a mixture of proteins through HPLC or isolated as gel spots in electrophoresis or blots. They still could be analytically demanding if the glycoproteins of interest are large biomolecules with low carbohydrate content (e.g., antibodies) or proteins glycosylated heavily at different sites. Yet more methodologically involved are the tasks where numerous glycosylated proteins are encountered in a complex biological medium, such as human physiological fluids or tissue extracts, at a very wide dynamic concentration range. These cases often present intricate analytical challenges. They represent an important direction in biomedical field. These types of studies will undoubtedly require selective pre-concentration of analytes together with the most sensitive and informative tools of analysis (Novotny and Mechref, 2005).

Glycoprotein is generally identified by PAS staining. This method has been applied to the direct demonstration of glycoproteins separated by SDS-PAGE (Segrest and Jakson, 1972). The method is quite specific, it is bundened by the following drawbacks: It requires considerable quantity of glycoprotein as it rather insensitive; it is time consuming and entails numerous washes (Gershoni *et al*., 1985). On the other hand lectin-based analyses can be quite sensitive and have effectively been applied to blots. However such procedures are restricted to the detection of specific sugar moieties (Gershoni and Plade, 1983; Clegg, 1982; Gershoni and Plade, 1982; Glass *et al*., 1981). Glycoproteins may be detected by enzyme hydrazides (Gershoni *et al*., 1985; Keren *et al*., 1986), fluorescent hydrazide (Steinberg *et al*., 2001), digoxigenin hydrazide or by autoradiography after incorporation of ^{3}H or ^{14}C sugars into cultures cells or tissues (Ivanov *et al*., 1998; Chandra *et al*., 1998).

Glycoconjugation using ^{125}I-tyrosine can be used for radioiodination of oligosccharides consisting proteins like antibodies and receptor etc. Radioiodination of antibodies and receptor using ^{125}I-tyrosine on to its sugar moiety could avoid iodination damaging and may increase the shelf-life of iodinated product. Background and non-specific binding can be reduced by radioiodination of glycoproteins using ^{125}I-Tyrosine conjugation method on to its sugar moiety. The method is simple, rapid and economic. The isolation and purification of glycoproteins from a complex protein sample and further step like western blotting were not required. MPD enhanced glycoprotein detection can be used as a tool for glycoproteome analysis in biomedical research.

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