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Fluorescent staining of glycoproteins in sodium dodecyl sulfate polyacrylamide gels by 4H-[1]-Benzopyrano[4,3-b]thiophene-2-carboxylic acid hydrazide

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Abbreviations

BH, 4H-[1]-Benzopyrano[4,3-b]thiophene-2-carboxylic acid hydrazide; PAS, periodate/Schiff’s base; DW, deionized water; EtOH, ethanol; MeOH, methanol; HAc, acetic acid; ACN, acetonitrile; ABC, ammonium bicarbonate; HIO₄, periodic acid.

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A sensitive, specific, economic and MS compatible staining method for gel-separated glycoproteins by using BH was described, meanwhile, the specificity of the newly developed stain for glycoproteins was demonstrated by 1-D and 2-D SDS-PAGE, deglycosylation, glycoprotein affinity enrichment and LC-MS/MS, respectively.

Abstract

A fluorescent detection method for glycoproteins in SDS-PAGE by using 4H-[1]-Benzopyrano[4,3-b]thiophene-2-carboxylic acid hydrazide (BH) was developed in this study. As low as 4-8 ng glycoproteins (transferrin, α1-acid glycoprotein) could be specifically detected by BH staining method, which is twofold more sensitive than that of the most commonly used Pro-Q Emerald 488 glycoprotein stain. Furthermore, the specificity of the newly developed stain for glycoproteins was demonstrated by 1-D and 2-D SDS-PAGE, deglycosylation, glycoprotein affinity enrichment and LC-MS/MS, respectively. According to the results, it is concluded that BH stain may provide new choices for convenient, sensitive, specific and economic visualization of gel-separated glycoproteins.
1. Introduction

Protein glycosylation has long been recognized as a very common post-translational modification, which is critical to growth control, cell migration, cell adhesiveness, tissue differentiation, and inflammatory reaction cascades [1-4]. To comprehensively understand a sophisticated biological system, researchers often need to determine if a preparation contains glycoproteins or if a protein is glycosylated.

Among the current strategies for glycoproteins study, SDS-PAGE is a reliable and commonly used technique for the separation, identification, and characterization of glycoproteins and glycoprotein mixtures [5, 6]. Several staining methods have been developed for the detection of gel-separated glycoproteins. Basing on the mechanism of selective glycoprotein detection, it mainly falls into two categories. One is based on affinity interaction between carbohydrate epitopes and lectin incorporated with reporter tags such as fluorescent or chemiluminescent substrates for downstream detection [7-9], usually applied to electroblot membranes. Another is to covalently react carbohydrate groups with hydrazine moieties through a periodate/Schiff base (PAS) mechanism, such as fuchsin [10], alcian blue [11], dansyl hydrazine [12], 8-aminonaphthalene-1, 3, 6-trisulfonate [13], Pro-Q Emerald 300 [14] and Pro-Q Emerald 488 [15]. The PAS-based approach can be used to label all kinds of gel-separated glycoproteins involving dialdehyde formation via oxidative cleavage of cis-vicinal diols on carbohydrates followed by the coupling of hydrazide reporters with aldehydes via Schiff base formation. Nowadays, one of the most commonly used visualization method for gel-separated glycoproteins is Pro-Q Emerald 488 stain, based on PAS mechanism, which could be commercially obtained from Invitrogen™.

Pro-Q Emerald 488 stain is a breakthrough technology that provides a simple and direct method for selectively staining glycoproteins. Though the sensitivity and specificity are significantly good, the problem of high costs for Pro-Q Emerald 488 stain has limited its application to high-throughput glycoproteomics in most laboratories.

Therefore, there is still necessary to provide a means of sensitive, specific, economic and MS compatible staining for gel-separated glycoproteins. Thus, 4H-[1]-Benzopyran-4,3-b-thiophene-2-carboxylic acid hydrazide (BH) was firstly introduced to detect glycoproteins specifically both in 1-D and 2-D SDS-PAGE in
the present work. As low as 4-8 ng glycoproteins could be specifically detected by BH staining method within 90 min, which is twofold more sensitive than the most commonly used Pro-Q Emerald 488 glycoprotein stain.

2. Experimental

2.1 Materials

Acrylamide, Bis, ammonium persulfate, Tris base, glycine, SDS, iodoacetamide, glycerol, bromophenol blue, ammonium bicarbonate (ABC), trypsin, BH, SYPRO Ruby, transferrin (glycoprotein, human, 80 kDa), BSA (bovine, 66 kDa), IgG (glycoprotein rabbit, 50 kDa), ovalbumin (glycoprotein, chicken, 45 kDa), α1-acid glycoprotein (glycoprotein, human, 41 kDa), α-casein (phosphoprotein, bovine, 25 kDa), β-casein (phosphoprotein, bovine, 24 kDa) and avidin (glycoprotein, egg, 16 kDa) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). CHAPS, DTT, PMSF, urea, IPG strip, cover oil, and IPG buffer were from Amersham Biosciences (Uppsala, Sweden). PNGase F (Cat # P0704L) was purchased from New England BioLabs (Beverly, MA, USA). Pro-Q Emerald 488 glycoprotein staining kit (Cat # P21875) was from Invitrogen™ (Carlsbad, USA). Glycoprotein Isolation Kit was purchased from Thermo Scientific™ (Thermo Fisher Scientific, Bremen, Germany). All other chemicals used were of analytical grade and were obtained from various commercial sources.

2.2 Solution preparation

BH stock solution (1%) was prepared with DMSO. Staining solution was prepared by diluting the BH stock solution 5000-fold into 1% acetic acid (HAc), just prior to use. The stock solution is stable for several months when stored in tightly sealed and foil-wrapped glass bottle at 4°C without decreasing the sensitivity of the staining results.

2.3 Preparation and separation of protein in 1-D SDS-PAGE

This study was formally approved by the Institutional Ethical Committee of the Second Affiliated Hospital, Wenzhou Medical University, and the human serum was obtained from seven healthy volunteers with informed
consent in the hospital. Proteins were prepared from human serum according to Pieper et al. [16]. Molecular weight marker proteins (transferrin, BSA, IgG, ovalbumin, α1-acid glycoprotein, α-casein, β-casein and avidin) and human serum total proteins were dissolved in a buffer containing 60 mM Tris (pH 6.8), 25% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.1% bromophenol blue for 1-DE. Prior to electrophoresis, protein samples were heated at 100°C for 5 min and then cooled to room temperature. Twofold serial dilutions of marker proteins and human serum total proteins were loaded onto the gel lanes, respectively. Electrophoresis was carried out on polyacrylamide slab gels (60×80×0.75 mm), using the discontinuous buffer system of Laemmli [17]. The 4.5% stacking gel was overlaid on the separating gel of 10% polyacrylamide with an acrylamide: Bis ratio of 30: 0.8. The running buffer consisted of 0.025 M Tris, 0.2 M glycine, and 0.1% SDS. The gels were run in a Mini-protein III dual slab cell (Bio-Rad, Hercules, CA, USA) at a constant current of 22 mA per slab gel using a Power PAC 300 (Bio-Rad).

2.4 Preparation and separation of protein in 2-D SDS-PAGE

For 2-D electrophoresis, the rats liver total proteins were separated using IPG strips (13 cm, linear 3-10 pH gradient). The samples mixed with rehydration buffer (8 M urea, 2% CHAPS, 2% IPG buffer, 0.04 M DTT, 1X nuclease solution, and a few grains of bromophenol blue) were rehydrated in an Immobiline DryStrip Reswelling Tray (GE Healthcare, Uppsala, Sweden). The rehydrated IPG strips were then transferred to the focusing tray and covered with mineral oil. IEF was undertaken using a horizontal electrophoresis Ettan IPGphor II system (Amersham Biosciences) by following conditions: instrument temperature 20°C; maximum 50 µA/strip; IEF step 1, 40 V for 12 h; step 2, 100 V for 4 h; step 3, 500 V for 2 h; step 4, 3000 V for 2 h; and step 5, 8000 V until the total volt hours reached 60 kVh. Upon completion of the first-dimensional electrophoresis, the IPG gel strips were removed from the Strip Holder. The excess mineral oil was allowed to drip from the IPG strip. Subsequently, strips were incubated in 10 mL of the first equilibration buffer (1% DTT, 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.1% bromophenol blue) for 15 min and 10 mL of the second equilibration buffer (2.5% iodoacetamide, 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.1% bromophenol blue) for 30 min.
SDS, 0.1% bromophenol blue) for 15 min prior to the second-dimensional electrophoresis. To transfer the IPG gel strips for the second dimension, the separating gel of 11.5% polyacrylamide with an acrylamide: Bis ratio of 30: 0.8 was cast using a Hoefer SE 600 system (Amersham Biosciences). IPG strips were then sealed on top of SDS-PAGE gels with agarose and run at a constant current of 22 mA per slab gel using power PAC 300 with running buffer.

2.5 Gel staining

2.5.1 BH stain

After electrophoresis, the gels were immersed in 100 mL of 40% ethanol (EtOH) and 10% HAc with gentle agitation for 30 min, followed by incubation in 100 mL oxidation buffer containing 0.5% HIO₄ and 1% HAc for 20 min. After that, the gels were washed three times for 5 min each in 1% HAc to remove excess HIO₄. Then, the gels were completely immersed in 100 mL 0.0002% BH and 1% HAc staining solution for 10 min. Finally, the gels were washed in 40% EtOH, 1% HAc and 1% ethanolamine destaining solution to remove unbound BH dye in the gel matrixs for 15 min. All steps were performed with a solution volume equivalent to ~10-fold excess of the gel volume. All staining and washing steps were performed with continuous gentle agitation in polypropylene dishes.

2.5.2 Pro-Q Emerald 488 stain

This staining method was essentially performed according to the instruction of Invitrogen™. After electrophoresis, the gels were incubated in 100 mL of 50% methanol (MeOH), 5% HAc fixing solution overnight to ensure that all of the SDS was washed out the gel. The gels were then washed two times in 3% HAc for 20 min each, and glycans were oxidized by incubation in 1% HIO₄, 3% HAc for 20 min. Then, the gels were washed three times for 20 min each in 3% HAc to remove the residual HIO₄. After that, the gels were immersed in Pro-Q Emerald 488 dye solution for 2 h. Finally, the gels were incubated in 3% HAc, three times for 30 min each and then rinsed briefly in DW.
2.5.3 SYPRO Ruby stain

The staining method was essentially performed according to Berggren et al. [18]. 2-D gels were fixed after electrophoresis using 10% MeOH, 7% HAc solution for 1 h; while, fixing was not required for 1-D gel before staining. 1-D gels were placed into 50 mL (300 mL for 2-D gel) SYPRO Ruby staining solution for no less than 3 h. Then the gels were rinsed in 10% MeOH, 7% HAc solution for 30 min.

2.5.4 EBT-silver stain

Eriochrome black T (EBT)-silver stain was essentially according to Jin et al. [19]. Briefly, after electrophoresis, gels were fixed in 200 mL of 40% EtOH, 10% HAc solution for 2×20 min. Gels were sensitized with 100 mL of staining solution containing 0.006% EBT, 30% EtOH solution for 2 min, and then destained using 200 mL of 30% EtOH solution for 2 min, and washed in 200 mL of water for 2×2 min. Gels were then impregnated in 100 mL of 0.25% silver nitrate containing 0.037% formaldehyde for 5 min, washed in 200 mL of water for 2×20 s, and immersed in 100 mL of 2% potassium carbonate, 0.04% sodium hydroxide, 0.007% formaldehyde, and 0.002% sodium thiosulfate solution to develop image. After silver ion reduction, gels were immersed in 100 mL of 1.5% EDTA for 10 min to stop the development.

2.6 Image analysis

The images stained with BH, Pro-Q Emerald 488 and SYPRO Ruby were generated using a Typhoon 9400™ scanner (Amersham Biosciences) with the resolution at 200 dpi. The image acquisition of silver-stained gels were digitized at 200 dpi resolutions using a scanner (UMAX PowerLook 2100XL, Umax Systems GmbH, Germany). The images were exported in TIF format and imported for analysis into Image Lab 2010 (Fujifilm Corporation, Japan) for 1-D gels and ImageMaster 2D Platinum v7.0 (Amersham Biosciences) for 2-D gels.

2.7 Fluorescence spectroscopy

To study the vitro photophysical properties of BH fluorescent dye, the excitation and emission spectra were recorded in MeOH at room temperature using a JASCO FP-6200/STR Fluorescence Spectrophotometer
JASCO International Co., Japan). The excitation spectrum was collected from 200 to 500 nm. For the emission profile, the excitation wavelength was set to 400 nm and the emission spectrum was collected from 300 to 600 nm.

2.8 Deglycosylation by PNGase F

The protocol used here was essentially according to the instruction of New England BioLabs. Briefly, 10 µg α1-acid glycoprotein or human serum total proteins were denatured with 1X Glycoprotein Denaturing Buffer (0.5% SDS, 40 mM DTT) at 100°C for 10 min. After the addition of NP-40 and G7 Reaction Buffer, two-fold dilutions of PNGase F were added and the reaction mix was incubated for 1 h at 37°C.

2.9 Glycoproteins enrichment by ConA affinity chromatography

To separate glycoprotein and non-glycoprotein fractions of human serum total proteins, lectin affinity chromatography was performed using a concanavalin A (ConA)–based Glycoprotein Isolation Kit. Briefly, unfractionated human serum containing up to 1.5 mg of total proteins were first diluted with the Binding/Wash Buffer and applied to the ConA resin bed. Following incubation for 10 min, the resin was washed and the bound glycoproteins were eluted.

2.10 LC-MS/MS

After detecting rats liver glycoproteins in 2-D gels with BH stain, interested glycoproteins were subjected to protease digestion and MS. Gel slices were destained with 3% HAc for 1 h. In-gel digestion was performed in three steps following the protocol modified by Russell et al. [20]. Briefly, the gel slices were washed with DW and 50% acetonitrile (ACN) and incubated with 100% ACN for 10 min. Opaque gels were equilibrated with 100 mM ABC (pH 8.0) for 5 min and then an equal volume of 100% ACN was added for 15 min. After removing the solution, the dried gel pieces were reduced with 10 mM DTT containing 100 mM ABC at 56°C for 45 min and alkylated with 55 mM iodoacetamide containing 100 mM ABC for 30 min in the dark. After removing the solution, the gels were washed with 100 mM ABC; and an equal volume of 100% ACN was added and
incubated. The gel pieces were dried in a SpeedVac for 15 min, and re-swollen by addition of trypsin solution and incubated overnight at 37 °C. Finally, peptides were subsequently extracted with 50% ACN/5% trifluoroacetic acid and dried in a SpeedVac. For the deglycosylation experiment, the tryptic peptides obtained from in-gel digestion were further deglycosylated by PNGase F in 50 mM ABC at 37°C for 2 h. Then, the samples were incubated at 75°C for 20 min to stop the reaction, and dried once again by using SpeedVac. To remove salt and contaminants from the peptide mixture, samples were purified with ZipTipC₁₈. In-gel tryptic digests (4 µL) were submitted to on-line nanoflow liquid chromatography using the EASY-nLC system (Proxeon Biosystems, Odense, Denmark, now part of Thermo Fisher Scientific) with 10 cm capillary columns of an internal diameter of 75 µm filled with 3 µm Reprosil-Pur C₁₈-A₂ resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The gradient consisted of 10-30% ACN in 0.1% formic acid at a flow rate of 200 nL/min for 45 min, 30-100% ACN in 0.1% formic acid at a flow rate of 200 nL/min for 1 min and 100% ACN in 0.1% formic acid at a flow rate of 200 nL/min for 10 min. The elution was electrosprayed through a Proxeon nanoelectrospray ion source by ESI-MS/MS analysis on a Thermo Fisher LTQ Velos Pro using full ion scan mode over the m/z range 200-1800. Collision-induced dissociation (CID) was performed in the linear ion trap using a 4.0-Th isolation width and 35% normalized collision energy with helium as the collision gas. Five dependent MS/MS scans were performed on each ion using dynamic exclusion. Also, the precursor ion that had been selected for CID was dynamically excluded from further MS/MS analysis for 30 s.

2.11 Data process and database search

The MS/MS spectra were processed using Proteome Discoverer (Version 1.3, Thermo Fisher Scientific - Waltham, USA) and the database search was performed using Mascot search engine (Matrix Science Mascot 2.3) against a concatenated forward-decoy approach. The Swiss-Prot protein sequence database (release 54.5) was searched, with taxonomy selection Rattus for sample. The search parameters were following: (1) two trypsin missed cleavage was allowed; (2) the mass tolerance was set at 1.5 Da for the precursor and 0.5 Da for the fragment ions; (3) carbamidomethyl (C) was chosen for fixed modification; (4) oxidation (M) and deamidated
(NQ) were chosen for variable modifications (herein, the variable modification of deamidated (NQ) was used to
determine the N-glycosylation sites); (5) proteins with scores above the significance threshold (P < 0.05) were
shown as significant hits. The hit with the highest score which contained at least two peptides with scores
beyond the identity threshold was regarded as the identified protein from each gel spot.

3. Results and discussion

3.1 Spectral characteristics of BH dye

As shown in Figure 1, fluorescence excitation/emission spectra of BH demonstrated that BH is optimally
excited at 398 nm, and has an emission maximum center around 430 nm.

3.2 Optimization of staining condition

The presence of buffer components in the gel matrixs, such as SDS and Tris, may interfere with the protein-dye
interaction. Thus, fixation is considered to be a critical procedure to remove buffer components in the gels,
which could greatly contribute to the interaction between BH dye and glycoproteins. Fixing of SDS-PAGE gels
in solutions containing MeOH or EtOH is known to remove much of SDS surrounding proteins. Therefore,
according to the results, total 30 min fixation in 40% EtOH, 10% HAc solution was recommended as an
efficient fixing condition in this study.

After fixing, cis-diol groups on glycoproteins were being oxidized to aldehydes by HIO₄ and covalently couple
to amine- or hydrazide-containing probe molecules during the selectively glycoprotein detection [21]. Thus, the
conditions introduced here (1% HIO₄ in 3% HAc, 20 min) were designed to ensure fully oxidation of all
glycoproteins with hydroxyl groups on adjacent carbon atoms. After that, the residual HIO₄ in the gel matrixs
was washed away by three changes of 1% HAc for 5 min each.

For the determination of the optimal BH concentration in staining solution, gels were stained with different
concentrations of BH ranging from 0.00005% to 0.001%. According to the results, 0.0002% BH gave the
highest fluorescence signal for glycoproteins with the lowest interference of non-glycoproteins. At lower or
higher concentration of BH dye, poor contrast and low sensitivity stains were obtained. In addition, to determine
the optimal composition of HAc in staining solution, gels were stained with the different concentration of HAc
ranging from 0.1% to 5% with 0.0002% BH for 10 min. Results showed that intense staining was obtained at
1% HAc. Moreover, to investigate whether the addition of certain substances might contribute to the increase in
the staining sensitivity, experiments to explore different additions of organic solvents (EtOH, MeOH, propylene
glycol, glycerol, and etc.), salt ions (aluminum, zinc, magnesium, sodium, and etc.), or buffering agents (Na$_3$PO$_4$,
Na$_2$HPO$_4$, borate acid, Tris, and etc.) to staining solutions were performed under the optimized staining
conditions discussed above. Nevertheless, better results were not achieved through modifications of the staining
solution. Therefore, the optimal constituent of staining solution was decided to be 0.0002% BH and 1% HAc.

Finally, to determine whether prolonged staining time affects the band intensity, gels were stained with 0.0002%
BH, 1% HAc staining solution for 10, 20, 40, 80, and 160 min, respectively. However, there was no increase in
the intensity between glycoprotein bands and background if the staining time is longer than 10 min. Thus,
staining the gels for longer than 10 min is not required.

For the specific detection of glycoproteins, rather surprisingly, HAc is another important constituent in destaining
solution to selectively distinguish glycoproteins from non-glycoproteins. On the basis of the various
concentrations of HAc, it was found that 1% HAc was appropriate for the specific detection of glycoproteins
considering sensitivity and a low background. Although the mechanism of HAc was not well understood, in the
present study, it was speculated that 1% HAc could provide a compatible pH value for the specifically
interaction between BH dye and glycoproteins.

**3.3 Protein detection in 1-D SDS-PAGE**

In the detection of serial dilutions of marker proteins ranging from 2 to 1000 ng, comparisons of the sensitivity
and specificity of BH stain with Pro-Q Emerald 488 stain were shown and summarized in Figure 2 and
Information Supporting Table 1. According to the results, we could clearly see that down to 4-8 ng of
glycoproteins (transferrin, α1-acid glycoprotein) could be detected by BH stain, which is approximately twofold
more sensitive than that of Pro-Q Emerald 488 stain that detected 8-16 ng of the same proteins. To confirm the
sensitivity and specificity, additionally, we have applied BH staining procedure to analyze human serum total
proteins (Figure 3). The results further demonstrated that the sensitivity and selectivity of BH stain is better than
that of Pro-Q Emerald 488 stain. Moreover, BH stain was carried out for around 90 min to complete all the
procedures compared with overnight protocol for Emerald 488 stain, indicating that BH stain is a time-saving
technique. Last but not least, the costs of BH and Pro-Q Emerald 488 stains have been evaluated carefully. For
per use, only 0.5 $ is enough for BH stain compared with 50 $ needed for Pro-Q Emerald 488 stain (mini gel),
indicating that BH stain is an economical staining method.

Furthermore, the effect of gel thickness on the detection of glycoproteins was evaluated in 0.5, 0.75, 1.0 and 1.5
mm thick SDS-PAGE. Gels of all thickness evaluated could readily be visualized with BH stain. However, 1.5
mm thickness gels couldn’t provide clear contrast between the glycoprotein bands and the background, leading
to a decreased sensitivity of staining. Operationally, 0.5–1.0 mm gels are superior for BH stain.

Moreover, for rational storage, BH stained gels were advised to be stored in DW at room temperature after
destaining, which could be stable for several hours without any decay on the sensitivity. On the other hand, the
fluorescent signal intensity of BH visualized glycoproteins remains constant even if the gels were exposed to
UV lamp (Molecular Imager Gel Doc XR, Bio-Rad) for about 1 h (data not show), which provides sufficient
time to address stained glycoproteins.

3.4 Application to 2-D SDS-PAGE

To evaluate the method critically, we have applied BH stain to provide a rational glycoproteomics map in 2-D
SDS-PAGE. The total proteins extracted from rats liver were separated, then stained with BH, Pro-Q Emerald
488 and SYPRO Ruby (Figure 4), respectively. Comparison of the spots visualized by the BH to those stained
by Pro-Q Emerald 488 showed that most of the spots visualized by Pro-Q Emerald 488 stain could be visualized
by BH stain. To further compare the sensitivity of BH stain and Pro-Q Emerald 488 stain, ImageMaster 2D
Platinum v7.0 was used to find and count protein spot numbers of the 2-D gels. After an automatic spot
detection, a number of 160 spots were detected using BH stain versus 139 spots detected with Pro-Q Emerald 488 stain. The results indicated that the sensitivity of BH stain is comparable or better than that of Pro-Q Emerald 488 stain as well.

3.5 Specific comparison

Specificity is one of the most important indices for the performance evaluation of glycoprotein staining methods. In order to investigate the specificity of BH stain, deglycosylation was performed on α1-acid glycoprotein (a representative glycoprotein) and human serum total proteins. The removal of glycosylation groups from these samples were conducted with PNGase F. As shown in Figure 5, in BH stained gels, deglycosylation-treated α1-acid glycoprotein and human serum total proteins showed extremely decreased band signal and band shifting, which could be observed in Pro-Q Emerald 488 stained gels as well. It is a kind of deglycosylated forms, to our known, caused by incomplete removal of deglycosylation groups on glycoproteins with PNGase F due to its substrate specificity. The results indicated that the specificity of BH stain is comparable with Pro-Q Emerald 488 stain.

In addition, lectin affinity enrichment is a widely used technique to isolate multiple types of glycoproteins from complex biological samples [21]. In this study, to further confirm the specificity of BH stain, ConA lectin Glycoprotein Isolation Kit from Thermo Scientific™ was introduced to enrich glycoproteins from human serum total proteins. As shown in Figure 6, the sensitivity and specificity of BH stain is comparable with that of Pro-Q Emerald 488 stain for glycoproteins analysis.

3.6 Reproducibility and linear dynamic range

Besides sensitivity and specificity, another main feature of important in large-scale proteome analysis is the reproducibility of the staining across the gels. In order to make statistical comparisons of stain properties, a database of 2-D gel images was created with three gels in each stain groups (BH stain and Pro-Q Emerald 488 stain). After spots detection and quantitation, a reference image was chosen to which all other images were matched by software transformation. The parameters used to determine the choice of the reference image were
overall quality of the image and number of spots. To simplify the graphs, a subset of the most accurately matched spots across all the gels was used to perform spot integrated intensity correlation. As shown in Supporting Information Figure S1, the average correlation of spot integrated intensities of BH stain ($r=0.937$) comes close to that of Pro-Q Emerald 488 stain ($r=0.896$), which indicated a similar reproducibility of both staining methods.

In addition, the linear dynamic range of glycoprotein detection was determined for BH stain and compared with Pro-Q Emerald 488 stain (Figure 7). In these gels, representative glycoproteins were serially diluted ranging from 2 to 1000 ng. The linear dynamic ranges of the amount of proteins stained with BH were for transferrin (4-1000 ng, correlation coefficient 0.994), α1- acid glycoprotein (4-1000 ng, 0.996), and avidin (4-1000 ng, 0.990), respectively. In general, the BH stain showed similar linear dynamic ranges to Pro-Q Emerald 488 stain with respect to the values of correlation coefficient of different glycoprotein density range.

### 3.7 Compatibility with MS

In order to determine the MS compatibility of the BH stain, the protein spots were excised from 3 replicated BH stained 2-D gels (Figure 4) for MS analysis. Totally 21 protein spots were selected and identified by MS following trypsin digestion and PNGase F deglycosylation (Table 1). The MS results suggested that BH stain did not interfere with the in-gel trypsin digestion toward BH-labeled glycoprotein and the downstream protein identification. In addition, the tryptic digestion of the BH stained spots were further deglycosylated by PNGase F, and the resulting peptides bearing N-glycosylation sites were then analyzed by LC-MS/MS (Table 1). Cleavage of oligosaccharides from glycoproteins catalyzed by PNGase F deaminates the linker as asparagines to aspartic acid, causing a mass shift of 1 mass unit [21]. The series of y ions from this peptide confirmed the match, and indicated that the 1-mass unit difference between asparagines and aspartic acid can be detected by LTQ Velos nano LC-MS/MS, thus confirming the precise glycosylation sites of the glycoproteins. For example, a representative deglycosylated peptide derived from catalase (spot 8 in Figure 4) was identified and its N-glycosylation site was assigned as N$_{436}$ (Figure 8), and totally 70 N-liked glycosylation sites were verified in the
21 identified glycoproteins.

4. Conclusions

In the present study, we confirmed that different glycoproteins could be specially labeled by BH staining method in 1-D and 2-D SDS-PAGE, and down to 4-8 ng glycoproteins could be selectively detected by BH in a short time, which is twofold more sensitive than that of the commonly used Pro-Q Emerald 488 stain. By understanding the advances and limitations of these the newly developed glycoprotein detection method, it could be concluded that BH stain would be plausible alternatives to the conventionally used Pro-Q Emerald 488 stain.

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References


Figure Legends

Figure 1. Chemical structure and fluorescence excitation/emission spectra of BH.

Figure 2. Comparison of the sensitivity and selectivity of different detection methods in 1-D SDS-PAGE with standard marker proteins. (A) BH stain; (B) ProTQ Emerald 488 stain; (C) SYPRO Ruby stain; (D) EBT-silver stain. The amounts of standard marker proteins in each lanes are as follows: lane 1, 1000 ng/band; lane 2, 500 ng/band; lane 3, 250 ng/band; lane 4, 125 ng/band; lane 5, 64 ng/band; lane 6, 32 ng/band; lane 7, 16 ng/band; lane 8, 8 ng/band; lane 9, 4 ng/band; lane 10, 2 ng/band.

Figure 3. Comparison of the sensitivity and selectivity of different detection methods in 1-D SDS-PAGE with human serum total proteins. (A) BH stain; (B) ProTQ Emerald 488 stain; (C) SYPRO Ruby stain. Lanes 1-10 (from left to right) were two-fold serial dilutions of human serum total proteins.

Figure 4. Comparison of the sensitivity and selectivity of different detection methods in 2-D SDS-PAGE with rats liver total proteins. (A) BH stain; (B) ProTQ Emerald 488 stain; (C) SYPRO Ruby stain. 21 protein spots were selected for further protein identification by in-gel trypsin digestion, PNGase F deglycosylation and LC-MS/MS.

Figure 5. Specificity comparison of BH stain with Pro-Q Emerald 488 and SYPRO Ruby stains for the detection of glycoproteins and deglycosylation treated proteins. (A) BH stain; (B) Pro-Q Emerald 488 stain; (C) SYPRO Ruby stain. Lane 1, nontreated total human serum proteins; Lane 2, PNGase F-treated human serum total proteins; Lane 3, nontreated α1-acid glycoprotein; Lane 4, PNGase F-treated α1-acid glycoprotein; Lane 5, PNGase F.

Figure 6. Specificity comparison of BH stain with Pro-Q Emerald 488 and SYPRO Ruby stains. (A) BH stain; (B) Pro-Q Emerald 488 stain; (C) SYPRO Ruby stain. Lane 1, 3 and 5, nontreated human serum total proteins; Lane 2, 4 and 6, eluted glycoprotein fractions from applied human serum total proteins by using Glycoprotein Isolation Kit.
Figure 7. Comparison of linear dynamic range between BH stain (A) and Pro-Q Emerald 488 stain (B). Three representative glycoproteins, transferrin, α1-acid glycoprotein and avidin were separated in a 10% polyacrylamide gel. After staining, the protein bands were estimated by software of Image Lab 2010. The range of amount of proteins tested was 4-1000 ng.

Figure 8. MS/MS spectrum of the deglycosylated peptide FNSANED\underline{N}VTQVR from spot 8 (catalase) in Figure 3. N represents the Asp residue formed via PNGase F deglycosylation from the glycosylation site at the Asn residue.
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A sensitive, specific, economic and MS compatible staining method for gel-separated glycoproteins by using BH was described, meanwhile, the specificity of the newly developed stain for glycoproteins was demonstrated by 1-D and 2-D SDS-PAGE, deglycosylation, glycoprotein affinity enrichment and LC-MS/MS, respectively.
(A) Transferrin $r=0.990$ (8-1000 ng)
- Avidin $r=0.987$ (8-1000 ng)
- $\alpha$ 1-acid glycoprotein $r=0.992$ (4-1000 ng)

(B) Transferrin $r=0.985$ (8-1000 ng)
- Avidin $r=0.980$ (8-1000 ng)
- $\alpha$ 1-acid glycoprotein $r=0.995$ (8-1000 ng)

77x85mm (200 x 200 DPI)