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Solid Phase Extraction of N-linked Glycopeptides Using Hydrazide Tip

Jing Chen¹, Punit Shah¹, and Hui Zhang¹

¹Department of Pathology, Johns Hopkins University, Baltimore, MD, USA

Abstract

Glycoproteome contains valuable information where biomarkers may be discovered for disease diagnosis and monitoring. Nowadays, with the ever increasing performances of mass spectrometers, the emphasis is shifting to the sample preparation for better throughput and reproducibility. Therefore, to facilitate high throughput N-linked glycopeptide isolation, in this study, a novel hydrazide tip was devised and an integrated workflow of N-linked glycopeptide isolation using hydrazide tips was presented. Using bovine fetuin as a standard glycoprotein, the incubation time was determined for each major step of glycopeptide isolation. Using commercially available human serum, multiple parallel isolations of glycopeptides were performed using hydrazide tips with a liquid handling robotic system. We demonstrated that, with the hydrazide tips, the processing time was significantly decreased from 3–4 days to less than 8 h with excellent reproducibility. The hydrazide pipette tips have great potential in achieving automation of N-linked glycopeptide isolation for high throughput sample preparation when used in combination with liquid handling robotic systems.

Keywords

Proteomics; glycopeptides; pipette tip; solid phase extraction; hydrazide

INTRODUCTION

Glycoproteins modified by oligosaccharides are expressed as transmembrane proteins, extracellular proteins, or proteins secreted to body fluids such as blood serum, which is an excellent source for diagnosis and monitoring of the presence and stage of many diseases ^{1,2}. As an easily accessible body fluid, human serum contains a large array of proteins that are derived from cells and tissues all over the body. Thus the human serum proteome contains valuable information where biomarkers may be discovered for clinical use, e.g. CA125 for ovarian cancer and PSA for prostate cancer ^{3,4}.

To analyze serum proteins, a robust method for isolation of glycopeptides using solid-phase extraction of N-linked glycopeptides from glycoproteins (SPEG) has been widely used ⁵.

This method isolates formerly N-linked glycopeptides containing glycosylation sites for N-

Correspondence to: Associate Professor, Hui Zhang, Johns Hopkins University, Department of Pathology, Clinical Chemistry Division, 1550 Orleans Street, Cancer Research Building II, Room 3M-03, Baltimore, MD 21231, Tel: 410-502-8149 (office), Fax: 443-287-6388, hzhang32@jhmi.edu.

glycans attachments and analyzes the peptides by mass spectrometry. Human serum N-linked glycoproteome is of special interest for a number of reasons ^{6,7}. First, by focusing on formerly N-linked glycopeptides, the complexity of the proteome is greatly reduced by only analyzing 1–2 N-glycosite containing peptides for each protein ⁸. Second, the high abundant non-glycoproteins, e.g. albumin that accounts for ~50% of proteins in human serum, are eliminated for mass spectrometry analysis. Third, glycoproteins account for most of the serum proteins that are derived from tissues where biomarkers may be identified. Fourth, aberrantly glycosylated peptides can be specifically isolated and analyzed using enrichment of glycopeptides with specific glycans ^{9,10}.

To this day, numerous studies have been carried out using the SPEG method for cancer biomarker discovery in serum and other body fluid including breast, ovarian, lung and liver cancers ^{11–14}. The SPEG method includes coupling of glycoproteins to a solid support using hydrazide chemistry and removal of non-glycoproteins, proteolysis of captured glycoproteins to hydrazide with trypsin, removal of digested non-glycopeptides with washing, and specific release of N-glycopeptides using peptide-N-glycosidase F (PNGase F). This procedure provides a straightforward work flow with good protein/peptide identification and specificity. However, the procedure requires a long processing time (4 days) ^{5,7} and is hard to scale up.

Nowadays, with the ever increasing performances of mass spectrometers, the emphasis is shifting to the sample preparation for better throughput and reproducibility. In addition, a greater than ever number of samples are being processed and subjected to mass spectrometry analysis, calling for automation for high throughput sample preparation. Automation can minimize variability due to human errors, provide greater consistency and reduce sample preparation time and effort. Therefore, to meet the pressing need in the mass spectrometry field, here we devised a novel hydrazide pipette tip and presented an integrated workflow of N-linked glycopeptide isolation using hydrazide tips. With the hydrazide tips, the processing time was decreased to less than 8 h. Furthermore, with the hydrazide tip, glycopeptide isolation could be automated using a liquid handling robot system.

EXPERIMENTAL SECTION

Materials

Hydrazide resin and sodium periodate were from Bio-Rad (Hercules, CA); BCA protein assay kit, Zeba spin desalting column (7k MWCO), Urea, tris(2-carboxyethyl) phosphine (TCEP) were from Thermo Fisher Scientific (Waltham, MA); sequencing-grade trypsin was from Promega (Madison, WI); PNGase F was from New England Biolabs (Ipswich, MA); alpha-CHC matrix was from Agilent Technology (Santa Clara, CA); frits were from POREX (Fairburn, GA); All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Preparation of Hydrazide Pipette Tip

A round frit (2-mm-diameter and 1-mm-thick, pore size 15–45 microns) were first pushed into the pipette tip end (Disposable Automation Research Tips, Thermo Fisher Scientific, Waltham, MA). Two hundred microliter of hydrazide resin (50% slurry) was then loaded into each pipette tip. Liquids were blown out of the tip and a 5 mm round frit was pushed

into the tip to secure the hydrazide resin between the two frits. The tips were then washed 5 times with 200 μ L water and conditioned 5 times with coupling buffer (100 mM sodium acetate, 1 M sodium chloride, PH=5.5) by aspirating and dispensing the solution. For less than 5% of the prepared tips, the flow was too slow due to high resistance, and the tips were therefore discarded.

Coupling Time for Glycoprotein to Hydrazide Tip

Four hundred microliter of bovine fetuin in oxidation buffer (500 mM sodium acetate, 0.3 mM sodium chloride, PH=5), was oxidized with 15 mM sodium periodate for 1 h at room temperature in the dark followed by buffer exchange into coupling buffer. After addition of 100 mM aniline, the fetuin samples were slowly pipetted through hydrazide tips for coupling. Aliquots of fetuin samples were saved before as well as after fetuin were coupled for 1, 5, 10, 20, 30, 60 and 120 min. Protein concentration was determined using BCA protein assay per manufacture's protocol after removal of aniline. The absorbance was read at 562 nm with a spectrophotometer (BioTek, Winooski, VT). The results were plotted against time and data presented represent mean $\pm$ SD (n=3).

Incubation Time for Trypsin Digestion

Bovine fetuin coupled to the hydrazide tips through oxidized glycans were washed with 3 mL urea buffer (8 M urea in 0.4M NH_4HCO_3), reduced with 10 mM TCEP for 30 min and alkylated with 12 mM iodoacetamide (IAA) for 15 min in the dark at RT. After washing again with 3 mL urea buffer, the conjugated fetuin was digested with trypsin (1:30) in 100 mM ammonium bicarbonate where the digested non-glycopeptides were released into trypsin solution. Aliquots of trypsin solutions were saved before and after the samples were digested for 1, 5, 10, 20, 30, 60 and 120 min. The peptide concentration in each aliquot was then determined by BCA protein assay. The results were plotted against time and data presented represent mean $\pm$ SD (n=3).

Incubation Time for PNGase F Release

After digestion, the hydrazide tips (with conjugated glycopeptides) were washed extensively with 6 mL solutions of 1.5 M sodium chloride, 80% acetonitrile (ACN), deionized (DI) water, and 25 mM ammonium bicarbonate buffer to remove any residual non-glycopeptides released by digestion. The hydrazide tips were then pipetted through 1500 U of PNGase F in 200 μ L of 25mM ammonium bicarbonate. Aliquots of PNGase F solutions (with released peptides) were saved before and after releasing for 1, 5, 10, 20, 30, 60 and 120 min. A 10^{-12} M angiotensin I standard in 50% ACN/1% TFA was used to serve as an internal standard. Equal amount of angiotensin I standard and samples (3 sets of fetuin glycopeptides collected at various time of PNGase F incubations) were applied to MALDI spots, coated with alpha-CHC matrix and analyzed by MALDI-TOF/TOF (4800, AB SCIEX, Framingham, MA). A total of 20 subspectra (100 shots/subspectrum) were averaged to yield the mass spectrum for each sample. Area under the curve for angiotensin I and the major fetuin glycopeptide released (LCPDCPLLAPLNSR) were recorded. The ratio of fetuin/angiotensin was calculated and plotted against time. Data presented represent mean $\pm$ SD (n=3).

Isolation of N-linked Glycopeptides from Human Serum with SPEG Using Hydrazide Tip

N-linked Glycopeptides was isolated from human serum using hydrazide tip similar to that described above. Briefly, 40 μ l of human serum (n=3) was diluted 1:1 with oxidation buffer, oxidized with sodium periodate and buffer exchanged into coupling buffer. The serum sample was then slowly aspirated into hydrazide tips and dispensed back into a 96-well plate for 30 min using a liquid handling robotic system (Versette, Thermo Fisher Scientific, Waltham, MA). The aspiration and dispensing were repeated during the entire incubation time. The glycoproteins captured in the hydrazide tips were then reduced, alkylated and digested by pipetting the tips through TCEP, IAA and trypsin solutions (1:120 based on initial protein amount, 1h). The tips were then washed extensively and glycopeptides were released with 1500 U PNGase F in 25 mM ammonium bicarbonate buffer for 1 h at RT. Tips were then washed 3 times with 50% ACN and The eluents were combined and vacuumed to dryness. Samples were resuspended with 40 μ l 5% ACN/0.2% formic acid. Two microliter of each sample was injected into Q-Exactive (Q-E, Thermo Fisher Scientific, Waltham, MA) for LC-MS/MS analysis.

LC-MS/MS Analysis

Formerly N-linked glycopeptides were analyzed using Q-E mass spectrometer with an EASY-Spray source. Peptides were separated with a 15 cm $\times$ 75 μ m C18 column on a Ultimate 3000 series UHPLC at a flow rate of 300 nL/min with a 110 min linear gradient (from 5 to 35% B over 75 min; A=0.1% formic acid 2% ACN in water, B=0.1% formic acid in 90% ACN). Full MS scans were acquired over the mass range 400–1800m/z with a mass resolution of 70,000. The AGC target value was set at 3,000,000. Fifteen most intense peaks were fragmented with Higher-energy collisional dissociation (HCD) with collision energy of 27. MS/MS was acquired with a resolution of 17,500 with an AGC target of 50,000 and max injection time of 200 ms. Dynamic exclusion was set for 15 sec.

Identification of Glycosites and Glycopeptide Quantification

The resulting MS/MS spectra were searched against the European Bioinformatics Institute (<http://www.ebi.ac.uk/>) non-redundant International Protein Index human sequence database (IPI, v3.87, 2011/09/27, 91,491 entries) using Proteome Discoverer (v 1.4, Thermo Fisher Scientific, Waltham, MA). Base peak profiles of the 3 LC-MS/MS replicates or the 3 isolation replicates were opened and overlaid in Xcalibur (Thermo Fisher Scientific, Waltham, MA). For peptide identification, a mass tolerance of 10 ppm was permitted for intact peptide masses and 0.6 Da for HCD-fragmented Ions, with allowance for two missed cleavages in the trypsin digests, oxidized methionine, and deamidated asparagine as potential variable modifications. Carboxyamidomethylation (C) was set as a fixed modification. Peptides with 1% FDR were reported with their peptide spectrum match (PSM). Peptides with N-glycosites (NXS/T, where X can be any amino acid except P) were required. For N-linked glycopeptides commonly identified in all 3 LC-MS/MS replicates or in all 3 isolation replicates, coefficient of variation (CV) for each peptide was calculated based on PSM; total PSMs was also calculated for each peptide by adding up the PSMs recorded in each run. The average CV for peptides with total PSMs over 150, between 150 and 60, between 60 and 30, between 30 and 15 and below 15 were calculated.

RESULTS AND DISCUSSIONS

Workflow of SPEG Using Hydrazide Tip

To achieve high throughput N-linked glycopeptide enrichment from serum, we devised a hydrazide tip for fast and reproducible N-linked glycopeptide isolation through solid phase extraction. Figure 1 shows the flowchart of N-linked glycopeptide isolation with hydrazide tips. Briefly, serum proteins with glycans oxidized were pipetted through hydrazide tips in the presence of 100 mM aniline where glycoproteins were conjugated covalently to the hydrazide resin packed in the tips. Glycoproteins captured to the tips were then denatured, reduced, alkylated and digested by aspirating and dispensing the hydrazide tips in urea, TCEP, IAA and trypsin solution respectively. The tips were then washed extensively with 1.5 M sodium chloride, 80% ACN, DI water, and 25 mM ammonium bicarbonate buffer to removed residual non-glycopeptides. Finally, the formerly N-linked glycopeptides were released by pipetting the hydrazide tips in PNGase solution.

Incubation Time for Each Step of SPEG Using Hydrazide Tip

To determine the reaction time of the major steps of SPEG, i.e. coupling, proteolysis and PNGase F release for glycopeptide capture of serum, bovine fetuin, a 38 kD glycoprotein with 3 N-linked glycosylation sites, was used as a standard.

To determine incubation time required for complete coupling, 0.8 mg oxidized bovine fetuin proteins were coupled with hydrazide tips in the presence of 100 mM aniline for various times. The amount of fetuin used here equals the amount of glycoprotein estimated from 40 μ l of human serum. Aniline was used here as a catalyst to improve the reaction rate between aldehyde and hydrazide groups as previously reported^{15,16}. We found that essentially no fetuin was present in solution at 10 min, suggesting that coupling was complete after 10 min incubation (Fig. 2A).

To determine the incubation time required for trypsin digestion, the fetuin proteins coupled to the hydrazide tips above were denatured, reduced, and alkylated. The fetuin samples were then digested with trypsin using a trypsin-to-glycoprotein ratio of 1:30 for various times. This trypsin-to-glycoprotein ratio was also used in the serum glycopeptide isolation where glycoproteins account for about 25% of the total serum proteins. We found that no additional peptides were released into the trypsin solutions after 1 h, suggesting that trypsin digestion was complete at 1 h (Fig. 2B).

To determine the incubation time required for PNGase F release of formerly N-linked glycopeptides, the hydrazide tips were washed extensively with 1.5 M sodium chloride, 80% ACN, DI water, and 25 mM ammonium bicarbonate buffer to remove any residual non-glycopeptides released by digestion. The hydrazide tips were then pipetted through PNGase F in 25 mM ammonium bicarbonate for various times. Again, the PNGase F-to-glycoprotein ratio is similar to that used in serum glycopeptide isolations. As shown in Figure 2C, most peptides were released after 1 h. At this time point, all three predicted formerly N-linked glycopeptides of fetuin (LCPDCPLLAPLNDSR, VVHAVEVALATFNAESNGSYLQLVEISR, and

RPTGEVYDIEIDTLETTCHVLDPTPLAN-CSVR) could be observed by MALDI-TOF-TOF (Fig. 2D).

Thus, with the hydrazide tip, the total time required to complete N-linked glycopeptide isolation is within 8h. The hydrazide tip contains hydrazide resins 40–60 micrometers in size with 0.1 μm macrospores. After packing, the spacing between resins is estimated to be roughly 50–90 micrometers considering a face-centered cubic or hexagonal close-packed arrangement¹⁷. Such small dimensions enable the tips to work as a microfluidic reactor, where reaction rate is significantly improved due to faster mixing¹⁸. As shown above, our method decreased the processing time to less than 8 hours. In addition, the isolation capacity could be easily adjusted by simply controlling the amount of hydrazide beads packed into each tip. As the loading capacity of the hydrazide beads is about 40 μl serum/200 μl hydrazide beads (50% slurry) as previously reported⁷, the hydrazide beads packed could be adjusted accordingly for optimal performance when a different amount of serum needs to be processed. Moreover, the workflow presented here could be used to isolate N-linked glycopeptides in body fluids other than protein samples. Finally, as the hydrazide tip could be readily used in liquid handling robotic systems, there are great potentials in the automation of N-linked glycopeptide isolation for high throughput sample preparation.

Rapid Analysis of N-glycoproteome of Human Serum

Subsequently, to attempt automation of isolation of N-linked glycopeptides, the hydrazide tips were used in combination with a liquid handling robotic system to perform glycopeptide isolation from human serum. Forty microliter of serum was processed with each hydrazide tip and 1/20th glycopeptide isolated was injected into Q-E for LC-MS/MS analysis. After controlling the FDR<1% for peptide identification, we identified 332, 345 and 328 unique formerly N-linked glycopeptides from human serum in 3 isolations respectively (Table 1). In comparison, similar number (315) of unique glycopeptides was identified from the same human serum when the isolation was carried out using the 4 day regular SPEG method⁵. The specificity of N-linked glycopeptides identified was also similar between the hydrazide tip isolations (89.04%, 86.59% and 90.07%) and the regular SPEG method (90.07%). The missed cleavages observed were 20.22%, 21.07% and 20.30%, respectively for the hydrazide tip isolations and was 16.38% for the regular SPEG method.

All together, a total of 379 unique formerly N-linked glycopeptides were identified in the 3 isolation replicates with 294 commonly identified (Fig. 3A) (Suppl. Table 1). Similarly, a total of 366 unique formerly N-linked glycopeptides were identified in the 3 LC-MS/MS replicates, with 306 commonly identified (Fig. 3B) (Suppl. Table 2). In both cases, the commonly identified peptides are about 80% of that totally identified. In addition, great consistency was observed in the LC profiles between the LC-MS/MS replicates and the isolation replicates (Suppl. Fig. 1). Furthermore, the reproducibility between isolation replicates was comparable to that between LC-MS/MS replicates, with CVs (based on PSMs) only slightly higher between isolations (Table 2). Overall, the CVs increased as the PSM of glycopeptides decreased as reported before¹⁹. The CVs between isolations were 6.32%, 11.36%, 9.98%, 17.01% and 28.1%, respectively, for glycopeptides with a total PSM over 150, between 150 and 60, between 60 and 30, between 30 and 15, and less than 15. In

comparison, the CVs between LC-MS/MS replicates were 4.53%, 6.27%, 8.57%, 11.53% and 21.55%, respectively, for glycopeptides with a total PSM over 150, between 150 and 60, between 60 and 30, between 30 and 15, and less than 15. These data demonstrate that glycopeptide isolation with hydrazide tips has high throughput, great reproducibility, and automation capability when used in combination with liquid handling robotic systems.

CONCLUSIONS

We have developed a hydrazide bead packed pipette tip for rapid, reproducible, and automated N-linked glycopeptide isolations. With the hydrazide tip, the processing time was significantly decreased from original 4-day-SPEG manual procedure to less-than-8 –hour automated process. In addition, we demonstrated that the hydrazide tip could perform glycopeptide isolations in a reproducible manner. Finally, we showed that the hydrazide tips are compatible with liquid handling robotics and have great potential in the automation of glycopeptide isolations for high throughput sample preparation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Workflow of formerly N-linked glycopeptide isolation using hydrazide tip.

Figure 2.

Determination of time required for coupling, trypsin digestion and PNGase F release. (A) Oxidized bovine fetuin was pipetted through hydrazide tip. Concentration of protein uncoupled was measured at various time points. (B) Fetuin conjugated to hydrazide tip was subjected to trypsin digestion. Concentration of non-glycopeptide released from glycoprotein conjugated on hydrazide tip was measured at various time points. (C) Fetuin glycopeptides conjugated to hydrazide tip through N-linked glycans were released by PNGase F. Peptide released was measured at various time points. (D) A representative MALDI spectra of formerly N-linked glycopeptides from fetuin. (S): Signal to Noise ratio of each peak.

Figure 3.

Venn diagram comparing the serum N-linked glycopeptide identified from 3 LC-MS/MS replicates and 3 isolation replicates. The diagram illustrates similarities and differences in the peptides identified in each of the 3 isolation replicates (A) and each of the 3 LC-MS/MS replicates (B) by proteome discoverer searches of MS/MS data.

Table 1

Identification, specificity and missed cleavage of glycopeptide Isolated using hydrazide tip and the original SPEG procedure. Formerly N-linked glycopeptides were isolated from 40 µl of human serum with SPEG method using hydrazide tip or with the original SPEG method; 1/20th glycopeptides isolated was injected into QE for LC-MS/MS analysis. The number and specificity of formerly N-linked glycopeptides identified as well as the percentage of peptides with missed cleavage was listed for each isolation.

Sample	Glycopeptides Identified	Specificity	Missed Cleavage
Hydrazide Tip Isolation 1	332	89.04%	20.22%
Hydrazide Tip Isolation 2	345	86.59%	21.07%
Hydrazide Tip Isolation 3	328	90.07%	20.30%
Original SPEG Isolation	315	81.66%	16.38%

Table 2

Reproducibility of Glycopeptide Isolations Using Hydrazide Tip. Formerly N-linked glycopeptides from 40 μ l human serum were isolated 3 times in parallel with SPEG method using hydrazide tip. 1/20th glycopeptides isolated from isolation #1 were injected 3 times into QE for LC-MS/MS analysis; 1/20th glycopeptides isolated from isolation #2 and #3 were injected once into QE for LC-MS/MS analysis. The MS/MS spectra generated were searched against human IPI 3.87 for identification of glycopeptides. Peptide spectral matches (PSMs) reported for each glycopeptide were used to calculate the CVs between injections and between isolations. The CVs were listed along with the total number of PSMs added up from each run.

No. of Total PSMs	Mean CV (%)	
	Between Injections (n=3)	Between Isolations (n=3)
PSM $\geq$ 150	4.53	6.32
150>PSM $\geq$ 60	6.27	11.36
60>PSM $\geq$ 30	8.57	9.98
30>PSM $\geq$ 15	11.53	17.01
15>PSM	21.55	28.1