

Specific Enrichment of N-linked Glycoproteins and Glycopeptides Using Hydrazine Chemistry

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1. Principle

Specific enrichment of N-linked glycoproteins and glycopeptides has recently been developed by Aebersold and his coworkers as an approach that is suitable for biomarker discovery. This approach consists of the following steps (Figure 1): (1) oxidation and covalent coupling: Carbohydrate groups of glycoproteins are oxidized into aldehydes by periodate and the oxidized glycoproteins are covalently coupled to hydrazide resin. No glycoproteins are removed by washing. (2) Proteolysis and Release of glycopeptides: Glycoproteins on the beads are digested by trypsin and non-glycopeptides are removed by washing and retained glycopeptides on the resin are released by PNGase F. (3) Released glycopeptides are analyzed by LC-MS/MS and LC-FTICR.

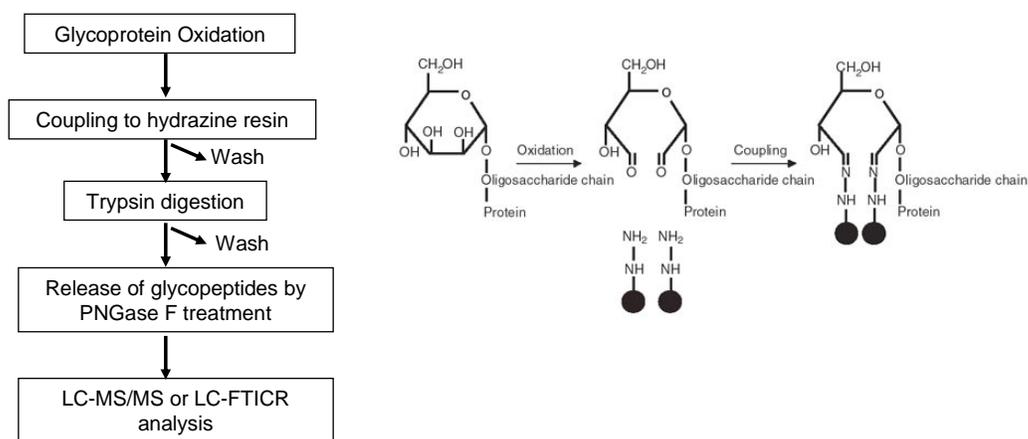


Figure 1. A schematic diagram of N-linked glycopeptide enrichment and analysis. Left: Strategy for the enrichment and analysis. Right: Oxidation of carbohydrate and hydrazide chemistry.

2. Protocols

- (1) Glycoprotein oxidation.** Complex protein samples such as human plasma are diluted 10 fold with coupling buffer (100mM Sodium Acetate, 150mM NaCl, pH 5.5) and sodium periodate is added to a final concentration of 15 mM. The oxidation reaction is carried out 1h at room temperature with shaking in the **dark**. After oxidation, the samples are desalted using PD-10 desalting columns (Amersham Biosciences).
- (2) Glycoprotein capture.** Hydrazine resin (Bio-Rad) (1mL dry resin per 5 mg proteins) is equilibrated with coupling buffer (3 washes with 1mL coupling buffer) in a 5 mL centrifuge tube. The oxidized proteins are desalting into the tube containing resin directly with the coupling buffer using PD-10 to remove NaIO₄ and other small MW sugars. The coupling reaction is carried out in 12-24 h with constant shaking. After coupling, supernatant (non-glycoproteins) is removed by centrifuging (3000 rpm, 1 min). The resin is further washed with 2mL urea soln. (8M urea, .4M NH₄HCO₃ pH 8.3) for 6 times to non-specific bound proteins.

- (3) **On-resin trypsin digestion.** The proteins on resin are denatured in equal volume of 8M Urea/0.4M NH₄HCO₃ for 1h at 37 °C and washing 3 more times with Urea. Proteins are then reduced by 10 mM DTT for 1h and alkylated by 40 mM iodoacetamide 1.5 h at 37 °C. After alkylation, resin is washed again with 8M Urea and 0.4 M NH₄HCO₃ for 3 times and 50 mM NH₄HCO₃ for three times. The resin is then diluted with 3 bed volume of 50 mM NH₄HCO₃ and sequencing grade trypsin is added at 1: 100 trypsin to protein ratio and the digestion is carried out overnight at 37 °C with constant shaking.
- (4) **Collection and removal of non-glycopeptides.** The trypsin release peptides in the supernatant (non-glycopeptides) is collected. The resin is further washed with equal volume of 2M NaCl (6X), 100% methanol (6X), and 0.1 M NH₄HCO₃ (6X) to remove non-specific bound peptides.
- (5) **Release of glycopeptides.** Glycopeptides are released from the resin with PNGase (1uL in 1ML buffer) at 37 °C overnight with constant shaking. The release peptides are collected and the resin is washed twice with 80% acetonitrile/0.1% TFA. All washes and the supernatant are combined and lyophilized. The sample is ready for LC-MS analysis.

Reference:

1. Zhang, H., Li, X.-j., Martin, D.B. & Aebersold, R. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol.* **21**, 660-665 (2003).