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Enrichment and Analysis of Non-enzymatically Glycated Peptides: Boronate Affinity Chromatography Coupled with Electron Transfer Dissociation Mass Spectrometry

Qibin Zhang[1], Thomas O. Metz[1], Ning Tang[2], Jonathan W. C. Brock[3], Heather M. Mottaz[1] and Richard D. Smith[1]

[1]Biological Sciences Division and Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99352

[2]Life Science & Chemical Analysis, Agilent Technologies, Santa Clara, CA 95052 [3]Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208

Overview

An efficient high-throughput method was developed for identifying low-abundance non-enzymatic glycated proteins in bottom-up proteomics, which couples phenyl boronate affinity chromatography and LC-MS/MS with electron transfer dissociation (ETD) as the fragmentation technique.

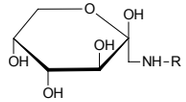
- Phenylboronate affinity chromatography can selectively enrich glycated proteins and peptides.
- Electron transfer dissociation can identify significantly more glycated peptides with higher confidence while preserving the labile modification group.

The combination of off-line phenyl boronate affinity enrichment and LC-MS/MS with ETD fragmentation may have broad applications in studies of diabetes mellitus.

Introduction

Non-enzymatic glycosylation or glycation – the covalent binding of single reducing sugars to primary amino groups – occurs in a time and sugar concentration dependent manner. The intermediate product is a stable, cis-diol containing ketoamine adduct called the Amadori Compound (AC), which may undergo further oxidation and/or rearrangement to form advanced glycation end-products (AGEs). The formation of AGEs on tissue proteins is believed to play a pathogenic role in the development of diabetic complications. Identifying the glycation sites on serum and tissue proteins that may lead to a pathological state is an important step towards finding more sensitive protein biomarkers for early diagnosis of diabetes mellitus and its associated complications.

Previous assays for protein glycation were unsuitable for glycation site identification. Because the total level of glycated proteins *in vivo* is only a few percent, it is necessary to have an enrichment step prior to MS analysis in bottom-up proteomics. An additional challenge in the analysis of glycated proteins is the identification of glycation sites by mass spectrometry. With collision-induced dissociation (CID), high-abundance ions corresponding to neutral losses of water dominate the MS/MS spectra, and poor sequence coverage with very limited peptide identifications are observed.[1]



AC from Glucose

Methods

Materials

Standard glycosylated proteins, *in vitro* glycated human serum, and RNase were purchased from Sigma, and Glycogel II boronate affinity gel was obtained from Pierce. RNase was glycated with D-glucose under anaerobic conditions for 3 and 14 days.[2]

Off-line boronate affinity enrichment

An empty Tricorn™ high performance liquid chromatography column (5mm x 100 mm) was packed with Glycogel II boronate affinity gel. Glycated proteins/peptides were then loaded onto the column and fractionated with an Agilent LC system equipped with a UV detector and fraction collector.

Mobile Phases: A, 50 mM $MgCl_2$ and 250 mM NH_4OAc, pH 8.1; B, 0.1 M HOAc.

Gradient: 0% B for 10 min; 0-100% B in 0.1 min; 100% B for 10 min; 100-0% B for 0.1 min; 0% B for 10 min.

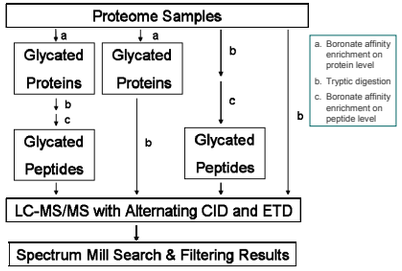
LC-MS/MS analyses of peptides

An Agilent 6340 Ion Trap LC/MS system equipped with ETD capability coupled with an Agilent HPLC-Chip Cube MS interface was utilized in analyses of peptides. Fluoranthene anions were used to transfer electrons, and the MS was operated in data-dependent MS/MS mode, with a full MS scan followed by 3 CID and ETD scans each (performed alternately).

Data analysis

The Spectrum Mill MS Proteomics Workbench was used for peptide identification by searching LC-MS/MS data against respective databases. The following criteria were used to filter raw search results: score > 8.0, %SPI > 60, Delta Rank1-Rank2 > 2.5.

Sample preparation and analysis flow-chart



Results

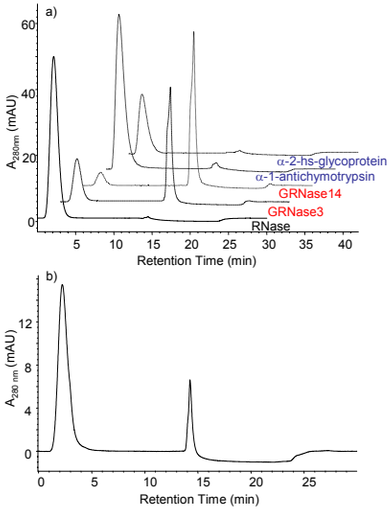


Figure 1. (a) Chromatograms of different standard proteins passing through the Glycogel II boronate affinity column; (b) Chromatogram showing the enrichment of glycated peptides from a tryptic digest of enriched glycated RNase. Peaks at ~ 2 min are non-glycated fractions, peaks at ~15 min are glycated fractions.

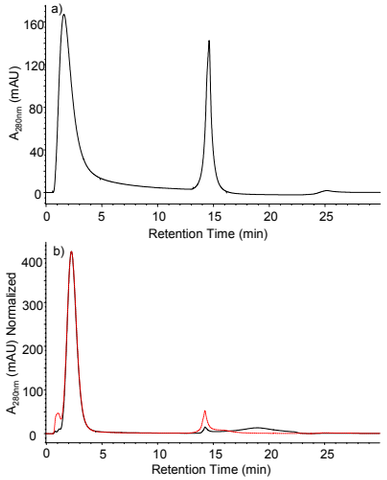


Figure 2. (a) Chromatogram showing the enrichment of glycated proteins from human serum glycated *in vitro*; (b) Chromatograms showing the enrichment of glycated peptides from tryptic digest of glycated proteins (dashed red line) obtained in panel (a) and non-enriched glycated human serum (solid black line).

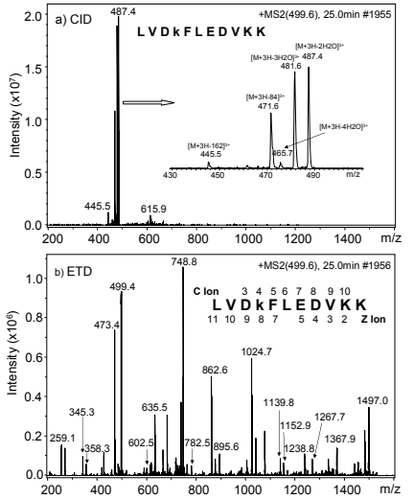


Figure 3. (a) MS/MS spectra obtained under CID (a) and ETD (b) fragmentation modes of m/z 499.6, the $[M + 3H]^{3+}$ of peptide LVDkFLEDVKK from α-1-antitrypsin precursor, in which "k" represents lysine modification with glucose. The identified c and z ions were labeled above and below the sequence.

Table 1. The unique glycated peptides identified from enrichment of *in vitro* glycated RNase at protein and peptide level. The lower case letters represent the modified amino acids.

Peptide Sequence	Glycation Site	Identified from Protein Enrichment	Identified from Peptide Enrichment
(-)KETAAAkFER(Q)	K^{7}	Yes	Yes
(R)QHMDSSTSAASSSNYcNQMMkSR(N)	K^{31}	Yes	Yes
(R)QHmDSSTSAASSSNYcNQMMkSR(N)	K^{31}		Yes
(R)ckPVNTFVHESLADVQAVcSQK(N)	K^{41}	Yes	Yes
(R)ETGSSkYPNcAYkTTQANK(H)	K^{91}, K^{98}	Yes	Yes
(R)ETGSSKYPNcAYkTTQANK(H)	K^{98}		Yes
(K)YPNcAYkTTQANK(H)	K^{98}	Yes	Yes
(K)TTQANkHIIVAcEGNPYVPVHFDASV(-)	K^{104}	Yes	Yes

Table 2. Summary of peptide identifications from different human serum peptide samples after LC-MS/MS analyses under alternating CID and ETD fragmentation modes. The number of peptide spectra identified from a reversed protein sequence database search appear in parentheses.

	Sample Name	CID total	CID-gly	CID-gly/ CID total	ETD total	ETD-gly	ETD-gly/ ETD total	FDR-CID	FDR-ETD
Binding Fractions	Dual Enrichment: Binding Fraction	88 (11)	15 (3)	17.0%	178 (5)	156 (2)	87.6%	11.1%	2.7%
	Peptide Level Enrichment only: Binding Fraction	63 (3)	10 (2)	15.9%	157 (7)	120 (1)	76.4%	4.5%	4.3%
	Protein Level Enrichment only: Binding Fraction	351 (21)	8 (7)	2.3%	252 (11)	31 (5)	12.3%	5.6%	4.2%
	Without Any Enrichment	386 (23)	3 (6)	0.8%	264 (3)	17 (0)	6.4%	5.6%	1.1%
Flow-through Fractions	Dual Enrichment: Flow-through Fraction	450 (29)	1 (8)	0.2%	206 (4)	0 (1)	0.0%	6.1%	1.9%
	Peptide Level Enrichment only: Flow-through Fraction	396 (22)	2 (4)	0.5%	226 (3)	0 (0)	0.0%	5.3%	1.3%
	Protein Level Enrichment only: Flow-through Fraction	356 (24)	4 (2)	1.1%	253 (4)	5 (0)	2.0%	6.3%	1.6%

Conclusions

- Boronate affinity chromatography selectively enriches glycated proteins/peptides in the presence of glycosylated species.
- Dual enrichment, with enrichment first on the protein level followed by enrichment on peptide level, is an efficient approach for enriching glycated peptides.
- Enrichment on the peptide level is more efficient than enrichment on the protein level when limited sample amounts are available.
- ETD can identify more glycated peptides with higher confidence compared with CID MS/MS analysis.
- Boronate affinity enrichment and LC-MS/MS with ETD fragmentation are very promising for studies of glycated proteins and peptides.

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Contact Information

Qibin Zhang, Ph.D.
Pacific Northwest National Laboratory K8-98
P.O. Box 999, Richland, WA 99352
e-mail: qibin.zhang@pnl.gov
Phone: (509) 373-9682