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Improved Methods for the Enrichment and Analysis of Glycated Peptides

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Abstract

Nonenzymatic glycation of tissue proteins has important implications in the development of complications of diabetes mellitus. Herein we report improved methods for the enrichment and analysis of glycated peptides using boronate affinity chromatography and electron-transfer dissociation mass spectrometry, respectively. The enrichment of glycated peptides was improved by replacing an off-line desalting step with an online wash of column-bound glycated peptides using 50 mM ammonium acetate, followed by elution with 100 mM acetic acid. The analysis of glycated peptides by MS/MS was improved by considering only higher charged (≥3) precursor ions during data-dependent acquisition, which increased the number of glycated peptide identifications. Similarly, the use of supplemental collisional activation after electron transfer (ETcaD) resulted in more glycated peptide identifications when the MS survey scan was acquired with enhanced resolution. Acquiring ETD-MS/MS data at a normal MS survey scan rate, in conjunction with the rejection of both 1+ and 2+ precursor ions, increased the number of identified glycated peptides relative to ETcaD or the enhanced MS survey scan rate. Finally, an evaluation of trypsin, Arg-C, and Lys-C showed that tryptic digestion of glycated proteins was comparable to digestion with Lys-C and that both were better than Arg-C in terms of the number of glycated peptides and corresponding glycated proteins identified by LC–MS/MS.

Nonenzymatic glycation of tissue proteins has important implications in the pathogenesis of diabetic complications[1] and in age-associated neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis.[2,3] Protein glycation begins with nucleophilic attack of a protein primary amine on the carbonyl group of a reducing sugar (glucose, glycolytic intermediates, etc.) to form a reversible Scruff base intermediate, which then rearranges to form a relatively stable ketoamine or Amadori adduct (Scheme 1). Under oxidative conditions, the Amadori adduct decomposes into more reactive carbonyl compounds that can further modify tissue proteins and alter their structure, function, and turnover rate.[4]

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SUPPORTING INFORMATION AVAILABLE

Table of unique glycated peptides and corresponding proteins identified from proteolytic digestion with trypsin, Lys-C, and Arg-C. This material is available free of charge via the Internet at http://pubs.acs.org.

Because glycation is a low-abundance, relatively nonspecific modification of proteins in vivo, we previously developed an enrichment method using boronate affinity chromatography to specifically enrich glycated peptides.[5,6] This method is highly specific and reproducible; however, an off-line peptide desalting step is required to remove the large amounts of salts collected together with eluted glycated peptides prior to downstream capillary liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. The off-line desalting step is not only laborious and time-consuming but can lead to extensive sample losses (up to 50%).

In addition to the low abundance of protein glycation in vivo, the Amadori adduct is also labile when analyzed by collision-induced dissociation (CID) MS/MS, readily losing up to 4 water molecules as neutral losses. In a recent study, we showed that electron-transfer dissociation (ETD) MS/MS does not result in these dehydration reactions and yields significantly better sequence coverage for glycated peptides.[7] ETD is distinct from CID in that labile side chain modifications on peptides remain intact, resulting in greatly improved backbone fragmentation and subsequent peptide identification.[8,9] We have applied ETD tandem mass spectrometry in proteomic analyses of glycated peptides from enzymatic digests of human plasma proteins and showed that ETD outperforms CID in the identification of glycated peptides.[5,6] However, ETD is not without its own limitations. For example, it is less effective for doubly charged ions and for ions with *m/z* over 850.[10,11] Two separate but similar approaches were recently developed to fragment charge-reduced nondissociative species: electron transfer followed by gentle supplemental collisional activation (ETcaD)[11] of the $[M + 2H]^{+\bullet}$ originating from doubly charged peptide precursors and CID of multiple charge-reduced species (CRCID).[12] Alternatively, it has been suggested that peptides cleaved by restrictive proteolytic enzymes other than trypsin may be more amenable to analysis by ETD, due to the generation of longer peptides with higher charge states.[9] Recently, a large number of phosphorylated peptides were identified by LC-ETD-MS/MS from yeast proteins treated with Lys-C.[8] In general, ETD is a relatively new technique, and additional studies are required to further realize the potential of this approach in the analysis of peptides containing labile modifications.

In this work, we sought to eliminate the off-line desalting step by introduction of an online wash immediately after glycated peptides were bound to the boronate affinity column. We also evaluated different data acquisition parameters (ETcaD, enhanced scan rate for better resolution in the MS survey scan, and selection of only highly charged precursor ions (≥3) for fragmentation) in order to improve the effectiveness of ETD for analysis of glycated peptides. In addition, we compared three different proteolytic enzymes (trypsin, Lys-C, and Arg-C) in digestions of glycated proteins from in vitro glycated human blood plasma.

EXPERIMENTAL SECTION

Chemicals and Materials

Human plasma samples from 20 healthy individuals were purchased from Bioreclamation, Inc. (Hicksville, NY) and received frozen on dry ice. All chemicals and peptide desalting SPE cartridges (Supelco Discovery DSC-18) were purchased from Sigma-Aldrich (St. Louis, MO), and the Micro-BCA protein assay kit was obtained from Pierce (Rockford, IL). Sequencing-grade trypsin was purchased from Promega (Madison, WI), while Arg-C and Lys-C were obtained from Roche Applied Science (Indianapolis, IN). Glycogel II boronate affinity gel (Pierce) was a gift from Dr. Bart Haigh of the Institute for Bioanalytics (Branford, CT).

Glycation of Human Plasma

Pooled plasma proteins (25 mg/mL) from 20 healthy individuals were incubated with 1 M glucose in PBS (pH 7.4) containing 1 mM diethylene triamine pentaacetic acid (DTPA) for 48

h at 37 °C. Glycated plasma proteins were then dialyzed overnight against PBS containing 100 mM DTPA using 3500 MWCO tubing.

Enrichment of Glycated Proteins by Boronate Affinity Chromatography

For optimization of boronate affinity chromatography, human plasma proteins glycated in vitro were used as model glycated species. Glycated human plasma was reconstituted in LC buffer A (vide infra) (1 μg/μL), and 500 μL was injected onto the Glycogel II boronate affinity column prepared in an empty Tricorn liquid chromatography column (5 mm × 100 mm; GE Healthcare, Piscataway, NJ). Chromatography was conducted on an Agilent 1100 series LC system (Agilent Technologies, Santa Clara, CA) equipped with a fraction collector. The LC buffers consisted of (A) 50 mM $MgCl_2$ and 250 mM NH_4OAc, pH 8.1, in water and (B) 0.1 M HOAc in water. A stepwise gradient separation totaling 30 min (0% B for 10 min; 0–100% B in 0.1 min, 100% B for 10 min; 100–0% B in 0.1 min; 0% B for 10 min) at 1.0 mL/min[5,6] was used as the control experiment for evaluation of buffers for online desalting. To optimize online washing of column-bound glycated proteins, the following volatile buffers were evaluated as part of a 10–15 min wash prior to elution of bound glycated proteins using 0.1 M HOAc: 100 mM NH_4HCO_3 and 25, 50, and 100 mM NH_4OAc, all pH 8.1. The LC effluent was monitored at 280 nm with a UV detector, and the quantification of glycated protein loss during the various online washes was calculated as the peak area corresponding to bound proteins (glycated) divided by the sum of the peak areas corresponding to bound and unbound (nonglycated) proteins. Each analysis was performed in duplicate.

The final optimized method was used to separate glycated from nonglycated peptides after digestion of glycated proteins with three different proteolytic enzymes, i.e., trypsin, Arg-C, and Lys-C. The fractions corresponding to glycated peptides were collected, dried in vacuo, and reconstituted without further processing for subsequent LC–MS/MS analysis.

Protein Digestion

Glycated plasma proteins were diluted in 8 M urea/100 mM NH_4HCO_3 (pH 8.0) to a concentration of 10 mg/mL. Disulfide bonds were reduced with 5 mM dithiothreitol for 1 h at 37 °C, followed by alkylation of free sulfhydryl groups with 20 mM iodoacetamide at 37 °C for 1 h in the dark. Samples were then diluted with 50 mM NH_4HCO_3 (pH 8.0) to reduce the urea concentration to below 1M. Alkylated proteins were digested separately with one of the following three endoproteases at a ratio of 1:67 (w/w, enzyme/protein): (1) for digestion with trypsin, $CaCl_2$ was added to a final concentration of 1.5 mM prior to addition of enzyme; (2) for digestion with Arg-C, an activation solution provided by the manufacturer was reconstituted with water and added to the sample such that the final dithiothreitol and EDTA concentrations were 5 and 0.5 mM, respectively. $CaCl_2$ was then added to a final concentration of 8.5 mM prior to addition of enzyme; and (3) for digestion with Lys-C, enzyme was added at the specified ratio without further adjusting the solution composition. All samples were digested overnight at 37 °C, and the peptide mixtures were passed through C_{18} SPE cartridges for desalting. Eluted peptides were dried in vacuo prior to boronate affinity enrichment of glycated peptides.

LC–MS/MS Analysis of Glycated Peptides

Glycated peptides were analyzed with an automated four-column capillary LC system coupled online with an LTQ-XL ion trap mass spectrometer (ThermoElectron, San Jose, CA) via an electrospray ionization interface operated in positive ionization mode.[13] For each analysis, ~2.5 μg of glycated peptides was loaded onto the reversed-phase capillary column (75 μm i.d. × 65 cm); the columns were slurry packed in-house with 3-μm Jupiter C_{18} particles (Phenomenex, Torrence, CA). The mobile-phase solvents consisted of (A) 0.2% HOAc and 0.05% TFA in water and (B) 0.1% TFA in 90/10 CH_3CN/H_2O. Exponential gradient elution was initiated 50 min after sample loading with a column flow rate of 400 nL/min, and the

mobile phase ramped from 0 to 60% mobile phase B over 100 min. The mass spectrometer was operated in data-dependent MS/MS mode with a precursor ion scan range of 350–1200 *m/z* (ions with *m/z* > 850 fragment poorly under ETD regardless of the precursor ion charge state,[10] which is consistent with unpublished results obtained in our laboratory from ETD-MS/MS analyses of peptides from tryptic digestion of *Shewanella oneidensis* proteins). ETD was used to fragment peptide precursor ions, and a dynamic exclusion of 60 s was applied to prevent repeated analyses of the same abundant precursor ions. The cation and anion populations were controlled by automatic gain control, and the target populations were set as follows: fluoranthene anions set to 1×10^5 and precursor cations from full MS scan events set to 3×10^4. The top 10 most intense ions from each MS scan were selected for fragmentation with the ion/ion reaction time fixed at 100 ms. Other parameters optimized during data acquisition were ETcaD, enhanced scan rate during MS scan events, and charge-state-dependent precursor ion rejection during MS^2 scan events. Each sample was analyzed on the same LC column in triplicate under each ETD-MS/MS data acquisition condition.

Data Analysis

To process the acquired data sets for identification of peptides, raw data files were searched with SEQUEST (ThermoElectron) against the human international protein index (IPI) database (Version 3.20 consisting of 61 225 protein entries) and its sequence-reversed database. Carbamidomethylation of cysteine (57.02 Da) was selected as a fixed modification, while Amadori compound modification at lysine (162.05 Da) and oxidation of methionine (15.99 Da) were selected as variable modifications for all searches. For those peptides generated by tryptic digestion, the requirement of at least partial tryptic peptide sequences was specified. For those peptides generated by Arg-C or Lys-C digestion, the amino acid immediately preceding the sequence or the last amino acid in the sequence was required to be arginine or lysine, respectively. Other SEQUEST search parameters were kept as default. The search results were filtered with the criteria outlined in Table 1, which resulted in a peptide false discovery rate of <0.8%. The Venn Diagram Plotter was used for plotting peptide overlap and is freely available at http://ncrr.pnl.gov/software/.

RESULTS

Optimization of Boronate Affinity Chromatography

We evaluated NH_4HCO_3 and NH_4OAc for washing column-bound glycated proteins free of salts. The rationale for choosing these volatile buffers was to minimize the presence of salt in collected glycated peptide fractions, which would be detrimental to downstream capillary LC—MS/MS analysis. In an initial evaluation, both buffers were prepared at 100 mM and pH 8.1 and applied as a 10-min wash prior to elution of glycated proteins. As shown in Figure 1a, washing with either buffer resulted in some loss of proteins, compared to the wash-free control. However, compared with the wash-free control experiment (33.3% recovery of proteins), the 100 mM NH_4HCO_3 wash resulted in a larger loss (27.0%) of proteins compared to the 100 mM NH_4OAc wash (30.4%). While the differences in protein loss described here and below were not dramatic, they were reproducible with coefficients of variation less than 1%, similar to our previously reported work.[5,6] Therefore, NH_4OAc was chosen for further evaluation as the wash buffer.

Three concentrations of NR_4OAc (100, 50, and 25 mM), all with pH 8.1, were subsequently evaluated to further optimize the wash step. The recovery of proteins after a 10-min wash with 100 and 50 mM NH_4OAc was essentially identical, 30.4 versus 30.3%, respectively (Figure 1b). However, the wash with 25 mM NH_4OAc reproducibly resulted in slightly lower (29.0%) recovery of proteins (Figure 1b). Therefore, 50 mM NH_4OAc was selected for further evaluation of the wash time.

Two wash times were evaluated with 50 mM NH_4OAc. As shown in Figure 1c, washing of column-bound glycated proteins with 50 mM NH_4OAc for 15 min resulted in more sample loss (28.5% recovery of proteins) compared to washing for 10 min (30.3% recovery of proteins).

The eluted glycated protein fractions from each of the above experiments were also collected and dried in vacuo to enable visual inspection of dried residue. The dried glycated protein fractions corresponding to the 50 and 25 mM NH_4OAc wash experiments both had negligible amounts of residue; however, the dried glycated protein fraction corresponding to the 100 mM NH_4OAc experiment contained visible salt residue. Therefore, in conjunction with the results from the wash time experiments, the final improved LC method for boronate affinity enrichment included a 10-min wash of column-bound glycated proteins with 50 mM NH_4OAc prior to their elution with 0.1 M HOAc. The improved LC method was then applied in the subsequent enrichments of glycated peptides obtained from in vitro glycaled human plasma after digestion with three different enzymes. The collected glycated peptide fractions were dried in vacuo, reconstituted in 0.1% TEA in water, and subjected to LC-ETD-MS/MS analyses.

Optimization of ETD-MS/MS Acquisition Parameters

We evaluated ETcaD, enhanced scan rate during MS scan events, and charge-state-dependent precursor ion rejection during MS^2 scan events in order to optimize ETD-MS/MS acquisition parameters for identification of glycated peptides from tryptic digests of human plasma proteins. Table 2 summarizes the results obtained from the evaluation of these data acquisition parameters. ETcaD in conjunction with the rejection of singly charged precursor ions resulted in the most identified spectra (3989) containing Amadori modification, but this did not translate into higher numbers of unique glycated peptides. In fact, this combination identified the least number (254) of unique glycated peptides among the four conditions evaluated. In contrast, ETcaD was more effective when combined with enhanced scan rate in the MS stage; this combination resulted in 346 unique glycated peptide identifications. The improved MS resolution provided by enhanced scan rate enables more accurate assignment of the precursor ion charge state. As a result, supplemental activation is performed more effectively. In general, ETcaD was amenable to glycated peptide analyses, since the neutral losses that typically dominate glycated peptide MS/MS spectra under CID conditions ($3H_2O$, $4H_2O$, $3H_2O$ + HCHO and $C_6H_{10}O_5$)[7,14,15] were insignificant in the ETcaD MS/MS spectra, due to the gentle collision energy (<20% normalized collision energy) applied in this process (Figure 2). However, when both singly and doubly charged precursor ions were rejected without ETcaD, the number of unique glycated peptide identifications increased further. Indeed, by just rejecting both singly and doubly charged ions without increasing the resolution for MS survey scans, the highest number of unique glycated peptides were identified (416).

We also evaluated the charge-state distribution for glycated peptides identified under the different data acquisition combinations described above in order to evaluate the effect of the enhanced scan rate function and the rejection of doubly charged precursor ions (Table 3). Rejecting only singly charged precursor ions coupled with ETcaD yielded a large number of doubly charged glycated peptides; as shown in Table 3, 39.8% of glycated peptides were identified as doubly charged if the normal precursor ion scan rate was selected. However, when the enhanced scan rate function was enabled, this value dropped to 22.2%. When both singly and doubly charged precursor ions were rejected, less than 2% of glycated peptides were identified as doubly charged. It is important to note that ~60% of the doubly charged glycated peptides could also be identified from their higher charged precursor ions in the two cases where ETCaD was enabled. In summary, the majority of the glycated peptides identified in

this study were triply charged, while less than 10% of the peptides carried four charges. Those peptides carrying five charges represented <1% of the identifications.

Evaluation of Different Proteolytic Enzymes for Analyses of Glycated Peptides

The glycated peptides obtained from digestion with Arg-C and Lys-C were analyzed in the same manner as those obtained from digestion with trypsin except with only one combined data acquisition parameter: enhanced MS survey scan rate plus rejection of both singly and doubly charged precursors. As shown in Table 2, the number of unique glycated peptides identified using Lys-C (353) was comparable to that identified using trypsin (382); however, the number of unique glycated peptides identified using Arg-C (183) was significantly less compared with the other two enzymes.

The charge-state distribution of identified glycated peptides (Table 3) revealed that Lys-C produced more glycated peptides capable of carrying four charges (16.9%) compared to trypsin (8.7%) or Arg-C (6.7%). The same trend was observed for glycated peptides carrying five charges, although, even with Lys-C, this number was relatively insignificant at 2.7%. Regardless of the enzyme used, the predominant charge state of identified glycated peptides was 3+, and <1% of the glycated peptides were doubly charged.

Among the unique glycated peptides identified from digestion of human plasma with the three enzymes, a significant overlap (124 peptides) existed between those peptides produced by digestion with trypsin and those produced by digestion with Lys-C (Figure 3a). As expected, there was very small overlap (11 peptides) between Lys-C and Arg-C, and 10 of these were the same among all three enzymes. In contrast, digestion with Lys-C resulted in more unique glycated protein identifications (81) compared to trypsin (78). Digestion with Arg-C again produced the fewest number of glycated protein identifications (47). Finally, there was significant overlap (28) in protein identifications among all three enzymes (Figure 3b).

DISCUSSION

It is known that pH and ionic strength of buffers can affect the binding of glycated species to boronate resin. For example, Litchfield et al. evaluated the effect of pH and optimized this parameter to 8.1 for maximal binding.[16] One goal of the present work was to introduce and optimize an online wash step during the enrichment of glycated peptides by boronate affinity chromatography in order to minimize sample loss during processing of clinical samples. The volatile buffers NH_4HCO_3 and NH_4OAc, both pH 8.1, were selected for this purpose due to their compatibility with downstream LC–MS/MS analyses. The observation of increased loss of glycated proteins when NH_4HCO_3 was used as the wash buffer cannot be explained at this time. It seems that ionic strength of the buffer, above a critical threshold, can maintain strong binding between glycated species and the boronate resin, which is demonstrated by the similar results obtained from the 100 and 50 mM NH_4OAc wash; however, 25 mM NH_4OAc is likely below this critical threshold, and the ionic strength is low enough to affect the binding of glycated proteins to the boronate affinity resin. There could be some loss of loosely bound glycated peptides by this procedure. likewise, this method may also eliminate some nonglycated peptides retained on the column. Regardless, the overall identification of glycated peptides was significantly improved (see below).

Several factors affect the efficiency of ETD, such as precursor ion charge-state, *m/z*, etc.[10,11] ETcaD can improve the fragmentation and sequencing of doubly charged peptide precursor ions by gentle collisional activation of their undissociated, charge-reduced species.[10,11] However, ETcaD is only applicable to doubly charged precursor ions; thus, its effective application requires that the charge state of the precursor ion be accurately determined. For this reason, ETcaD coupled with enhanced scan rate during the MS survey scan resulted in

more unique glycated peptide identifications than when coupled with a normal scan rate. However, ETcaD did not outperform the rejection of both singly and doubly charged precursor ions during data-dependent MS^2, in terms of glycated peptide identifications (Table 2). This observation is related to the fact that peptides are typically present in more than one charge state in the gas phase and that, in the current study, the majority of the glycated peptides (~60%) identified by doubly charged precursor ions were also identified by triply charged precursor ions. Thus, doubly charged ions that may dominant the MS spectra will inhibit the selection of other less abundant yet highly charged ions for fragmentation. Further, ETcaD requires more time to complete, thereby decreasing the duty cycle of the analysis. For example, we calculate that ~20% less scans are acquired when ETcaD is enabled. Therefore, simply excluding doubly charged precursor ions from selection for fragmentation results in higher numbers of glycated peptide identifications compared to ETcaD.

To determine the charge state of a precursor ion during the MS survey scan in LTQ ion trap instruments, there are three common scan options: normal scan, enhanced scan, and zoom scan. The option with the highest mass resolution and accuracy in assigning the precursor ion charge state is the zoom scan. However, the long scan time (910 μs/u) renders the zoom scan impractical for high-throughput data-dependent LC–MS/MS proteomics applications. The enhanced MS survey scan (intermediate rate between zoom scan and normal scan) option used in the current study is faster and less accurate, but can still assign charge states up to 3+ more accurately than the normal scan rate (60 μs/u). However, our results show that this advantage is limited when combined with the exclusion of both singly and doubly charged precursor ions from fragmentation. Further, this scanning option requires more time to complete, decreasing the analysis duty cycle. As shown in Table 3, 0.5% of peptides still were doubly charged even when the enhanced scan rate function and rejection of doubly charged precursors were both enabled. In comparison, 1.6% of peptides were doubly charged when a normal scan rate was used, suggesting that the error rate of assigning doubly charged ions is low even with a low-resolution linear ion trap mass spectrometer operating under a normal rate of MS survey scan. In general, the normal scan rate is the fastest but least accurate option for determining the precursor ion charge state. However, it can assign 1+ and 2+ charge states fairly effectively.

Overall, for the four data acquisition conditions we have evaluated, an average of 85% of the identified peptides contained the Amadori modification at lysine, illustrating the high specificity of the improved boronate affinity enrichment method.

It has been suggested that the proteolytic enzymes Arg-C and Lys-C are more compatible with ETD analyses because they generally generate longer peptides.[9] However, our results indicate that trypsin was comparable to Lys-C in terms of the number of unique glycated peptide and unique glycated protein identifications. Both enzymes outperformed Arg-C. These observations are likely attributable to the Amadori modification on lysine—trypsin does not cleave C-terminal to Amadori-modified lysine.[17,18] Thus, it will generate longer peptides than for proteins not carrying modifications at lysine. However, we cannot explain why digestion with Arg-C resulted in fewer glycated peptide identifications. Since trypsin has been shown to work very well in bottom-up proteomic analyses of glycated proteins,[5,6,19] and because it is more affordable than both Lys-C and Arg-C, it is the preferred choice.

CONCLUSIONS

We demonstrate that an online wash of column-bound glycated proteins with 50 mM NH_4HCO_3 for 10 min is a viable high-throughput method for minimizing the presence of salts in eluted fractions. In addition, acquiring ETD-MS/MS data at a normal MS survey scan rate, in conjunction with the rejection of both 1+ and 2+ precursor ions, can increase the number of identified glycated peptides, relative to ETcaD or an enhanced MS survey scan rate. Finally,

proteolytic digestion with Lys-C or Arg-C does not offer advantages over digestion with trypsin, in regard to the total number of identified glycated peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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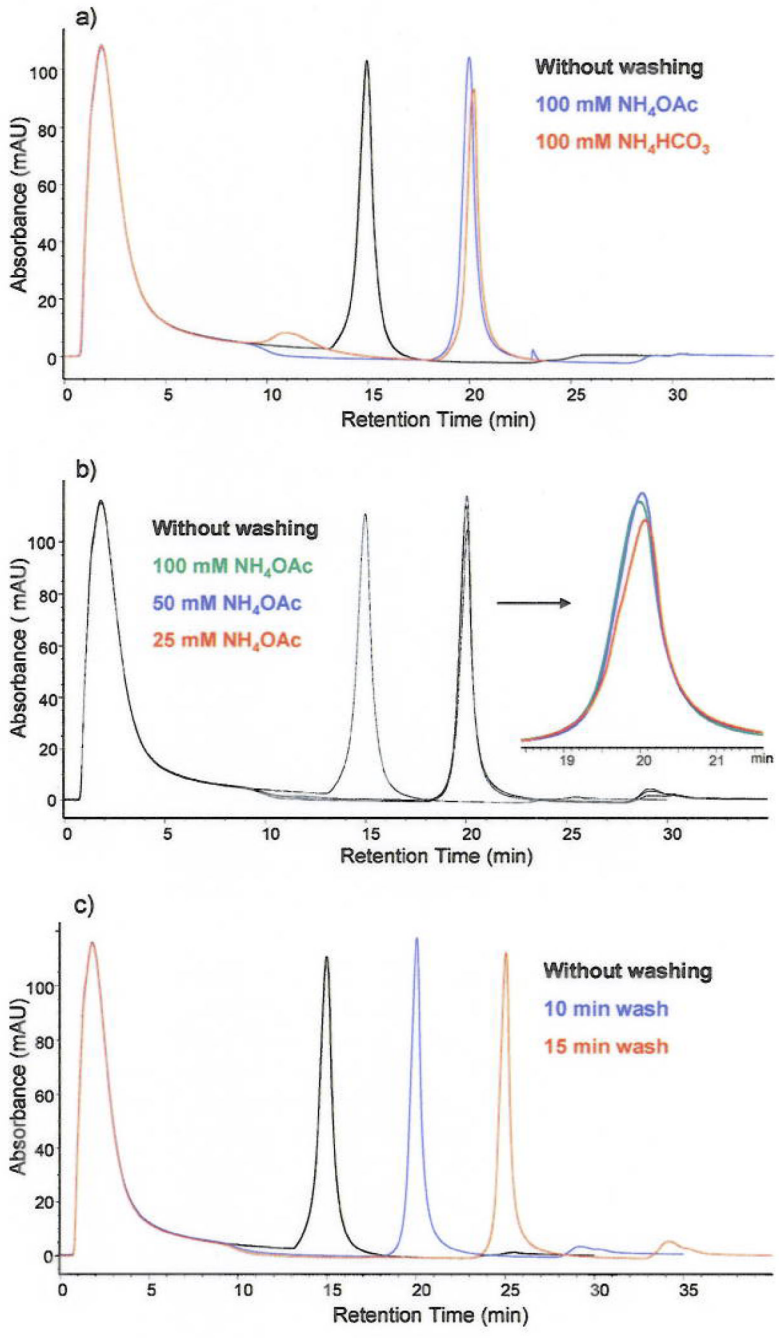


Figure 1.

Boronate affinity chromatograms. Peaks at 2 min are eluted nonglycated proteins, and peaks at 15, 20, and 25 min are glycated proteins eluted after a 0-, 10-, or 15-min wash, respectively. The same amount of in vitro glycated human plasma was injected onto the boronate affinity column in each experiment. (a) Effect of 10-min wash with different buffers; (b) effect of 10-min wash with different concentrations of NH_4OAc (inset is zoomed view of binding fractions); and (c) effect of different wash times with 50 mM NH_4OAc on the recovery of glycated proteins.

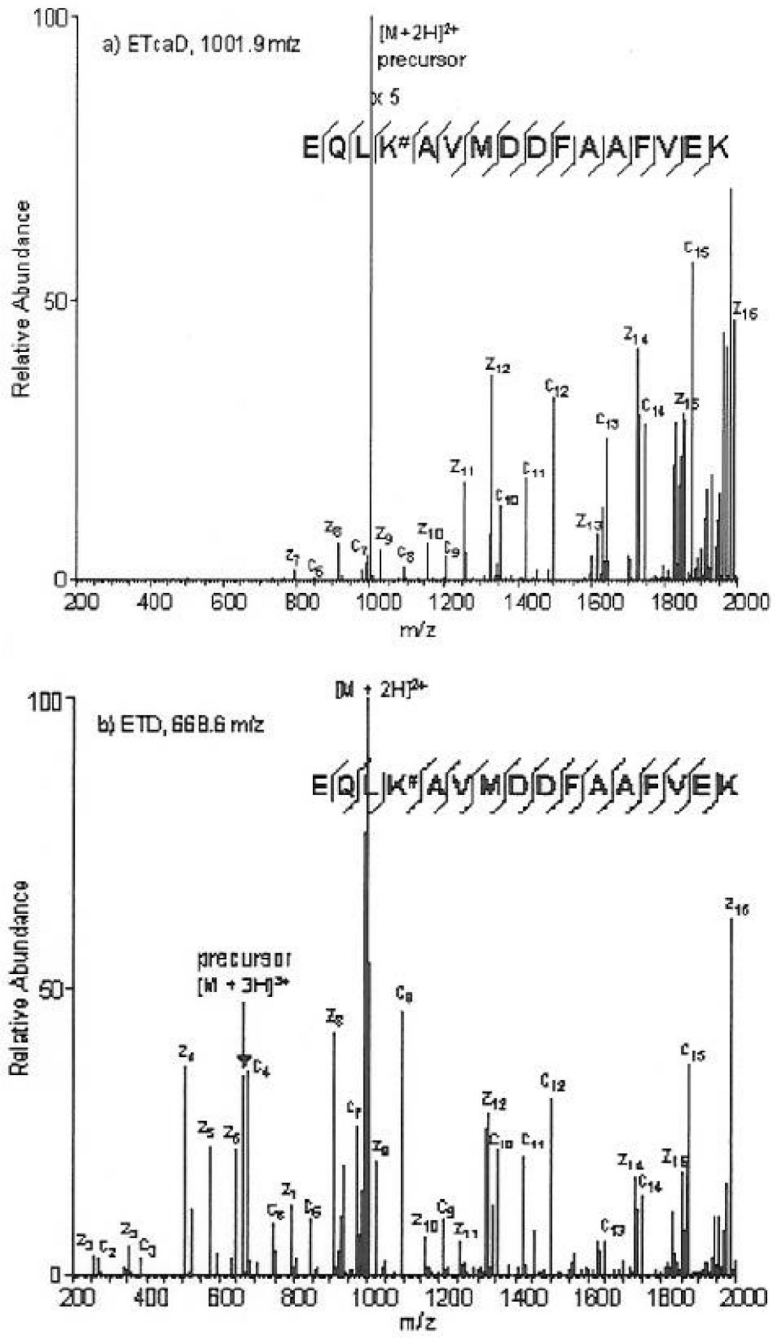


Figure 2.

Product ion spectra produced by ETcaD (a) and ETD (b) MS/MS analysis of peptide EQLK#AVMDDFAAFVEK, where # represents the site of the Amadori adduct modification. Both spectra were acquired during data-dependent LC–MS/MS analysis of the same sample. Comparison of the data obtained with ETcaD to that obtained with ETD indicates that ETcaD is amenable to the analysis of glycated peptides.

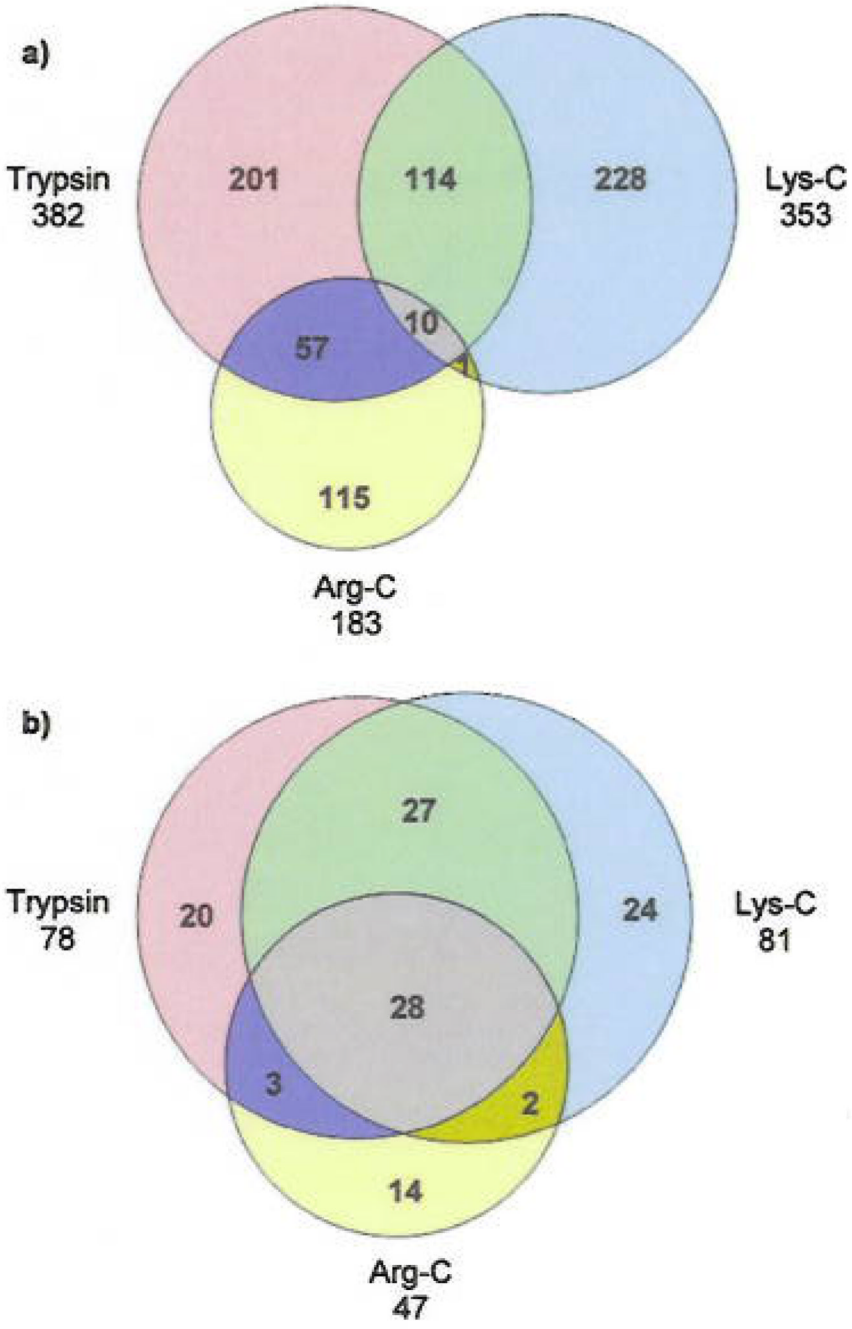
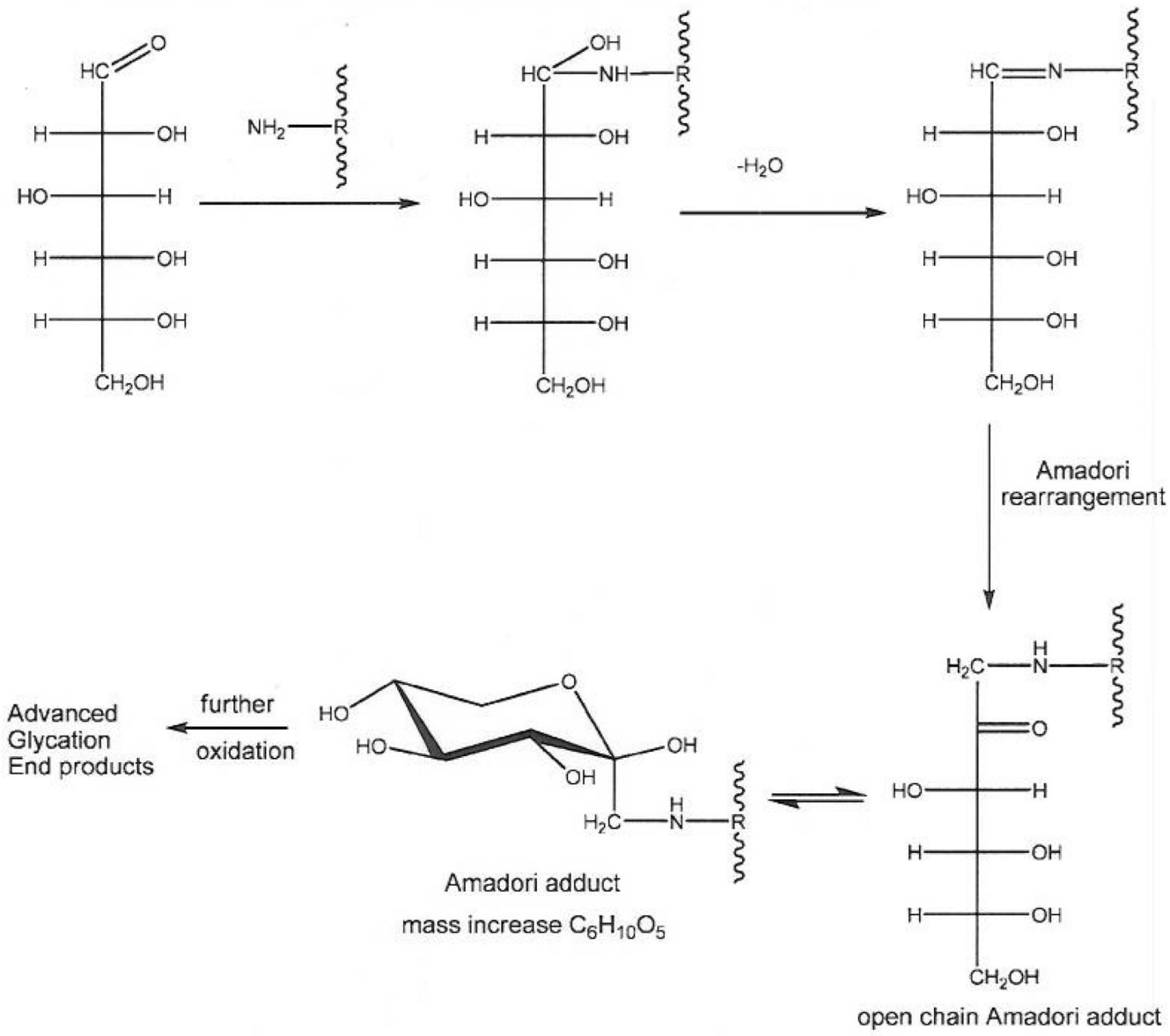


Figure 3.
Venn diagrams showing overlaps between (a) unique glycated peptides and (b) unique glycated proteins. Numbers correspond to unique glycated peptides or unique glycated proteins identified after digestion with trypsin, Arg-C, and Lys-C.



Scheme 1.
Formation of the Amadori Adduct from D-Glucose and Primary Amine

Table 1

Criteria for Filtering SEQUEST Results[a]

charge state	Xcorr	DelCN2	cleavage type
2	≥1.9	≥0.1	fully tryptic
2	≥3.0	≥0.1	partially tryptic
3	≥2.6	≥0.1	fully tryptic
3	≥4.0	≥0.1	partially tryptic
4	≥3.5	≥0.1	fully tryptic
4	≥4.7	≥0.1	partially tryptic
5	≥4.3	≥0.1	fully tryptic
5	≥5.7	≥0.1	partially tryptic

[a]The estimated false discovery rate for identified peptides was below 0.8% for each charge state using these filtering criteria.

Table 2

Results Obtained by Variation of ETD-MS/MS Data Acquisition Parameters and of Proteolytic Enzymes[a]

digestion enzyme	data acquisition parameter setup				total no. of spectra	total no. of modified spectra	unique modified peptides
	ETcaD	enhanced scan	reject 1+ ion	reject 2+ ion			
trypsin	Y	Y	Y	N	2619	2177 (83.1%)	346
	Y	N	Y	N	4716	3989 (84.6%)	254
	N	Y	Y	Y	2585	2224 (86.0%)	382
	N	N	Y	Y	2785	2402 (86.2%)	416
Arg-C	N	Y	Y	Y	1619	1397 (86.3%)	183
Lys-C	N	Y	Y	Y	3064	2637 (86.1%)	353

[a] Y, enabled; N, disabled. Number in parentheses represent identified spectra containing glycated peptides and are expressed as a percent of total identified spectra. “Modified” indicates peptides containing Amadori modification at lysine.

Table 3

Charge-State Distribution for Identified Glycated Peptides[a]

digestion enzyme	data acquisition parameter setup				total no. of modified peptides	charge-state			
	EtcaD	enhanced scan	reject 1+ ion	reject 2+ ion		2+	3+	4+	5+
trypsin	Y	Y	Y	N	2177	484 (22.2%)	1454 (66.8%)	221 (10.2%)	18 (0.8%)
	Y	N	Y	N	3989	1586 (39.8%)	2328 (58.4%)	74 (1.9%)	2 (0.1%)
	N	Y	Y	Y	2224	11 (0.5%)	2006 (90.2%)	194 (8.7%)	13 (0.6%)
	N	N	Y	Y	2402	38 (1.6%)	2109 (87.8%	237 (9.9%)	18 (0.7%)
Arg-C	N	Y	Y	Y	1397	9 (0.6%)	1265 (90.6%)	94 (6.7%)	29 (2.1%)
Lys-C	N	Y	Y	Y	2637	16 (0.6%)	2106 (79.9%)	445 (16.9%)	70 (2.7%)

[a]Number in parentheses represent identified glycated peptides for the given charge-state and are expressed as a percent of total identified glycated peptides across all charge-states. “Modified” indicates peptides containing Ama-dori modification at lysine.