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Chemo-Enzymatic Detection of Protein Isoaspartate Using Protein Isoaspartate Methyltransferase and Hydrazine Trapping

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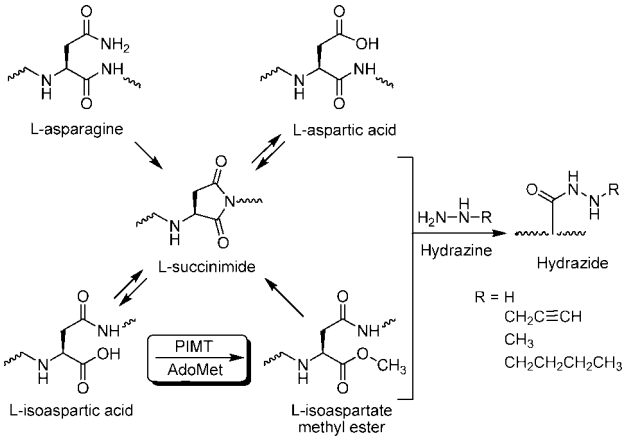
Isoaspartate formation is a ubiquitous post-translation modification arising from spontaneous asparagine deamidation or aspartate isomerization. The formation of isoaspartate inserts a methylene group into the protein backbone, generating a "kink", and may drastically alter protein structure and function, thereby playing critical roles in a myriad of biological processes, human diseases, and protein pharmaceutical development. Herein, we report a chemo-enzymatic detection method for the isoaspartate protein, which in particular allows the affinity enrichment of isoaspartate-containing proteins. In the initial step, protein isoaspartate methyltransferase selectively converts isoaspartates into the corresponding methyl esters. Subsequently, the labile methyl ester is trapped by strong nucleophiles in aqueous solutions, such as hydrazines to form hydrazides. The stable hydrazide products can be analyzed by standard proteomic techniques, such as matrix-assisted laser desorption ionization and electrospray ionization mass spectrometry. Furthermore, the chemical trapping step allows us to introduce several tagging strategies for product identification and quantification, such as UV−vis and fluorescence detection through a dansyl derivative. Most significantly, the hydrazide product can be enriched by affinity chromatography using aldehyde resins, thus drastically reducing sample complexity. Our method hence represents the first technique for the affinity enrichment of isoaspartyl proteins and should be amendable to the systematic and comprehensive characterization of isoaspartate, particularly in complex systems.

Isoaspartate (isoAsp) formation is a ubiquitous nonenzymatic post-translational modification arising from spontaneous asparagine (Asn) deamidation or aspartic acid (Asp) isomerization via succinimide intermediates,[1–3] as depicted in Scheme 1. The formation of isoAsp inserts a methylene group into the protein backbone, generating a "kink", and thus may drastically alter protein structure and function. In most cases, isoAsp formation is considered as protein damage; on the other hand, it may also play a role in signaling and regulation, perhaps acting as a molecular clock.[2,4,5] IsoAsp formation has been implicated in a wide range of diseases, such as autoimmunity and enhanced amyloid formation.[6,7] Significant isoAsp accumulation is also commonly observed in protein reagents and pharmaceuticals, affecting their efficacy and toxicity.[3] Yet isoAsp is certainly even more prevalent in biological systems and plays much broader roles

Scheme 1. Isoaspartate Formation, Protein Isoaspartate Methyltransferase (PIMT)-Catalyzed Methylation, and Trapping of Methyl Ester and Succinimide with Hydrazines



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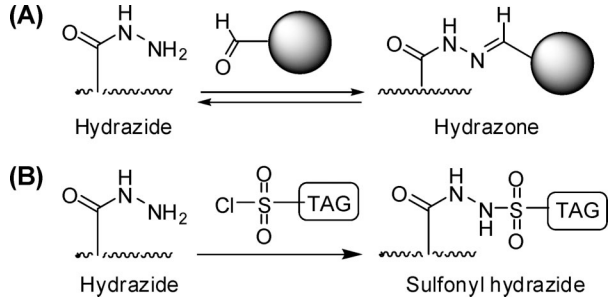
as well. For instance, analysis of the human genome reveals that approximately half of human proteins contain at least one Asn-Gly sequence, which is most prone to deamidation with a half-life as short as 1 day under physiological conditions.[4,8] Accordingly, there is growing interest in characterizing isoAsp in both biological research and pharmaceutical discovery. Progress in these fields has been hampered by the lack of sensitive and specific detection methods for isoAsp, particularly in complex samples.

Currently, isoAsp is detected by immunological, instrumental, or enzymatic methods.[3,9–11] For example, high-performance liquid chromatography (HPLC) or isoelectric focusing (IEF) separation combined with mass spectrometry (MS) are most commonly used.[12] Recently, O'Connor and co-workers were able to detect isoaspartyl residues in proteins by electron capture dissociation (ECD) MS.[9–11] Due to the subtle differences in mass and physical properties between Asn and isoAsp (mass difference of 1 Da and charge difference less than 1 depending on the pH) and aspartate (Asp) and isoAsp (no change in mass and net charge), effective separation and identification of isoaspartate-containing proteins and peptides by instrumental methods is often tedious and laborious. Alternatively, high specificity toward isoAsp is achieved through an enzymatic method using protein isoaspartate *O*-methyltransferase (PIMT or PCMT, EC 2.1.1.77),[1,13] a process commercialized by Promega under the name IsoQuant. As illustrated in Scheme 1, the PIMT enzyme methylates isoAsp to the corresponding methyl ester using *S*-adenosylmethionine (AdoMet) as the methyl donor. As a result, the presence and location of isoAsp can be deduced from the isoaspartyl methyl esters. However, the isoaspartyl methyl esters are labile and spontaneously cyclize to form succinimides, which can then hydrolyze to isoAsp or Asp (see Scheme 1). Since methyl ester cleavage can be rapid with half-lives as short as 4 min, the intrinsically labile methyl ester and succinimide are not reliable tags for detection.[14] As such, none of these existing methods have demonstrated the capability of analyzing complex systems. Furthermore, most of the isoAsp in cellular proteins are present in only a small fraction of each polypeptide, making sensitive detection and effective enrichment methods all the more critical.[1–3]

We report herein a chemo-enzymatic approach for isoAsp detection, by trapping the labile isoaspartyl methyl esters and succinimides with nucleophilic hydrazines or hydroxylamines as shown in Scheme 1. The resulting peptidyl hydrazides are stable and can be directly detected and sequenced using standard LC and MS. Moreover, several options are available to facilitate comprehensive characterization, as illustrated in Scheme 2. For example, stable isotope tags can be easily introduced for relative quantification or determination of isoaspartate location by MS, similar to detection of succinimide by labeling with ^{18}O water or

Scheme 2. (A) Affinity Enrichment of Hydrazide with Aldehyde Resin. (B) Hydrazide Labeling with Tags Containing Chromophores or Fluorophores



methylation products by deuterated AdoMet.[15,16] Additionally, hydrazides can be selectively derivatized with aldehyde or sulfonyl chloride tags that contain chromophores or fluorophores, allowing identification and absolute quantification by techniques other than MS. Most significantly, peptidyl hydrazides can be affinity-enriched with aldehyde resins with high efficiency and selectivity, offering a practical method for large scale analysis of complex systems. Our approach, hence, represents the first method enabling affinity enrichment of isoaspartate containing peptides and proteins.

EXPERIMENTAL SECTION

General Methods and Procedures. All aqueous solutions were prepared in water purified by a Milli-Q purification system (Millipore, Bedford, MA). The pH was measured on a PerpHecT LogR meter, model 320 (Orion, Boston, MA), using a PerpHecT ROSS Combination pH electrode, model 8202BN (Orion, Boston, MA) or a Semimicro PerpHecT ROSS Combination pH probe, model 8203BN (Thermo Electron, Beverly, MA). The pH levels for solutions less than 1 mL in volume were checked using Colorphast pH indicator strips with a pH 5−10 range with 0.5 pH unit accuracy (EMD). All incubations were carried out in a water bath at 37 °C unless specified otherwise. Adenosyl-homocysteine nucleosidase (MTAN) was prepared as previously described.[17–19] Human recombinant PIMT (rhPCMT isozyme II, EC 2.1.1.77) was at a concentration of 0.27 mg/mL (11 μM) in a pH 8 solution containing 5 mM sodium phosphate, 5 mM EDTA, 0.1 mM DTT, and 10% glycerol.[20] The specific activity of the enzyme at 37 °C was 21 000 pmol methyl transferred/min/mg protein or 5.6 pmol/min/μL protein. Sequencing-grade modified trypsin was purchased from Promega (Madison, WI). Peptide stock solutions were prepared in water, and the concentrations were determined by UV light at 280 nm using the extinction coefficient 1215 M^{-1} cm^{-1} for tyrosine and 5630 M^{-1} cm^{-1} for tryptophan.[21,22] Peptide

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stock solutions were stored at 4 or −20 °C. β-delta sleep-inducing peptide (DSIP, Trp-Ala-Gly-Gly-isoAsp-Ala-Ser-Gly-Glu) was purchased from Bachem America (Torrance, CA). KASA-isoD-LAKY was synthesized as a trifluoroacetic acid (TFA) salt by California Peptide Research, Inc. (Napa, CA). *S*-Adenosylmethionine *p*-toluenesulfonate, ^{14}N and ^{15}N hydrazine dichloride, *N*-methylhydroxylamine hydrochloride, phenylmethylsulfonyl fluoride (PMSF), ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), periodate-treated agarose (P3568), and bovine brain calmodulin (P0809, crude and P2277, 95% pure) were purchased from Sigma-Aldrich (St. Louis, MO). Dansyl chloride and methyl hydrazine were from Acros (Morris Plains, NJ) and Fisher Scientific (Pittsburgh, PA). Propargyl hydrazine was synthesized according to a literature procedure.[23] The acetohydrazide used was from TCI (Portland, OR). All reagents were ACS grade or finer and used without further purification. Hydrazine (2 M) stock solutions were prepared from the dichloride salts, and the pH was adjusted to 7.5−8.1 using NaOH. *S*-Adenosylmethionine (AdoMet, 4.69 mM) was prepared in 1 mM HCl, and the concentration was determined by absorption at 260 nm using the extinction coefficient 15 400 M^{-1} cm^{-1}.[24] **Caution***: Hydrazines are commonly used reagents and we did not experience any safety issues; nevertheless, highly concentrated and anhydrous hydrazines should be handled with care. In our hands, aqueous solutions of hydrazines (up to 4 M) were stable for over several months when stored in a closed container and at or below room temperature.*

Aging of Calmodulin. IsoAsp was generated based on a literature procedure reported by Aswad's laboratory.[25] Briefly, bovine brain calmodulin (P2277 from Sigma, 95% pure, ∼1 mg) was dissolved in 0.5 mL of 50 mM HEPES and 1 mM EGTA at pH 7.4 to give a final concentration of 279 μM as determine by UV absorption at 280 nm using the extinction coefficient 2980 M^{-1} cm^{-1}, estimated by ProtParam on the ExPASy server.[21,22] The protein solution was aged by incubating at 37 °C for 15 days, then stored at −20 °C. In addition, to test our procedures with protein mixtures, crude calmodulin was also used. Briefly, calmodulin (P0809 from Sigma, crude, 2.3 mg) was dissolved in 1 mL of 50 mM HEPES and 2 mM EGTA at pH 7.3 to give a final concentration 137 μM by weight. The sample contained roughly equal amount of S-100 proteins and calmodulin as estimated by matrix-assisted laser desorption ionization-MS (MALDI-MS) analysis (see Figure S1.1 in the Supporting Information). Thus, the concentration of calmodulin was estimated to be 69 μM, which was used for the subsequent experimental report. The sample was incubated at 37 °C for 25 days and then stored at −20 °C.

Tryptic Digestion of Calmodulin. Aged crude calmodulin solution (89 μg, 77 μL of a 69 μM solution by weight) was mixed with an equal volume of Tris−HCl (100 mM, pH 8.3) followed by the addition of trypsin (10 μg, 15 μL of 0.67 μg/μL solution in 50 mM acetic acid, pH 3.5) each hour for 2−2.5 h at 37 °C followed by the addition of PMSF (1.7 μL of a 100 mM solution in absolute ethanol) to a final concentration of 1 mM to quench the reaction. Tryptic digests were then filtered using Microcon YM-10 filters with a molecular weight cutoff of 10 000 Da from Millipore (Bedford, MA). Aged 95% calmodulin (358 μg, 77 μL of 279 μM solution) was digested using the same method as above. The final concentration for calmodulin crude was estimated to be 30 μM, which accounts for there only being 50% calmodulin based on MS analysis. The final concentration for 95% calmodulin digest was 122 μM. Tryptic digests were stored at −20 °C until subsequent labeling with PIMT and hydrazine.

One-Pot Methylation and Hydrazinolysis of Aged Calmodulin. To 3.5 μL of 100 mM Tris−HCl at pH 8.3, the following were added in the order listed: AdoMet (312 μM, 1 μL of a 4.69 mM solution), aged calmodulin digest (14 μM, 7 μL of a 30 μM crude calmodulin solution aged 25 days), adenosylhomocysteine nucleosidase (0.35 μM, 0.5 μL of a 10.5 μM working solution), and PIMT (2.2 μM, 3 μL of an 11 μM stock solution). Adenosylhomocysteine nucleosidase was added to alleviate product inhibition, as we previously reported.[17−19] The resulting solution was immediately mixed with equal volumes of ^{14}N hydrazine (2 M, pH 7.4) or ^{15}N hydrazine (2 M, pH 7.5−8.0). The final concentrations of reagents and enzymes were as follows: 156 μM AdoMet, 7 μM peptide digest, 0.18 μM adenosylhomocysteine nucleosidase, 1.1 μM PIMT, and 1 M hydrazine. Reactions were then incubated at 37 °C for 7−8 h. Following the same procedure, calmodulin 95% (28 μM final concentration) treatments were prepared as described above. The negative controls were prepared without PIMT using identical conditions as treatments. The rationale for one-pot labeling, where the methylation reaction was carried out in the presence of hydrazine, was to maximize hydrazide yields by the immediate trapping of newly formed methyl esters and succinimide, thus minimizing loss due to hydrolysis. In addition, any methyl ester or succinimide hydrolyzed to isoAsp is methylated again for another opportunity to be trapped with hydrazine.

Sample Preparation for Affinity Enrichment of Peptidyl Hydrazides. Calmodulin (95% pure, aged 15 days, 28 μM, 100 μL) underwent tryptic digestion, methylation, and hydrazinolysis using the one-pot method described above. The negative control was prepared using identical conditions as the treatment without PIMT. Samples were desalted after the labeling reaction to remove hydrazine using Extract Clean C18 solid-phase extraction (SPE) cartridges of 100 mg bed weight from Alltech (Deerfield, IL). The peptide digest reaction mixture was directly loaded to the cartridge and washed with 3 × 250 μL of 0.1% (v/v) aqueous TFA. The samples were then eluted from SPE cartridges using 5 × 200 μL of 25/75 (v/v) 0.1% aqueous TFA/acetonitrile. Elutions were combined and then evaporated to dryness under vacuum at 37 °C for 18 h on a CentriVap concentrator from Labconco (Kansas City, MO). The peptides were then reconstituted in 42 μL of sodium acetate (100 mM, pH 5) prior to binding to affinity columns.

Construction of Column for Affinity Enrichment. Affinity enrichment was performed using columns constructed by our laboratory containing periodate-treated agarose beads. The column format was adapted to avoid bead and sample loss, which was problematic when working with suspended beads of small-scale quantities. The columns were constructed from 10 μL pipet tips with the ends tightly crimped to prevent elution and bead loss while standing. The resin used for affinity enrichment was periodate-treated agarose (Sigma, P3568, 60−75 nmol aldehyde

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equivalents per μL of packed beads according to the manufacturer). To reduce nonspecific binding, beads were washed three times with acetohydrazide (227 mM, pH 3, pH adjusted with TFA) prior to loading the columns. During each wash, the beads were allowed to stand for 15 min with acetohydrazide. Then, the beads were washed three times with sodium acetate (100 mM, pH 5). The beads in sodium acetate (50% volume packed beads) were stored at 4 °C. The columns were loaded with 5 μL of suspension of the pretreated beads (~2.5 μL packed beads), and the storage buffer was eluted via centrifugation (6000 rpm, 2000 RCF) with a VWR Galaxy mini centrifuge from VWR (West Chester, PA). Centrifuge adapters purchased from Glygen (Columbia, MD) were used to suspend columns above 1.5 mL centrifuge tubes during centrifugation.

Affinity Enrichment of Calmodulin Hydrazides. The packed columns were loaded with an aliquot of calmodulin hydrazide solution (20 μL) in sodium acetate (100 mM, pH 5) as prepared above. The columns were then placed in sealed 1.5 mL centrifuge tubes to reduce evaporation and allowed to stand 14 h at room temperature (rt). The binding solution was then eluted, and the columns were washed with 5 × 20 μL sodium acetate (100 mM, pH 5). Hydrazides were eluted from the columns using 10 μL of acetohydrazide (227 mM, pH 3). During the elution process, the eluent was allowed to stand in the column for 15 min and then recycled two additional times. The eluted solutions were desalted using Zip Tips prior to MS analysis.

Dansylation of Peptide Hydrazides. We investigated the selectivity of dansylation of hydrazides against amines by modifying the KASA-isoD-LAKY ^{14}N hydrazide, a peptide with two lysine amines and a free N-terminal amine, with dansyl chloride under mildly acidic conditions (pH 1.5−2). The KASA-isoD-LAKY peptide was labeled using a stepwise procedure where methylation was performed in a solution containing 50 μM peptide, 156 μM AdoMet, 0.18 μM MTAN, and 0.55 μM PIMT in 100 mM potassium phosphate, pH 7.0, in a total volume of 1000 μL and incubated for 6 h at 37 °C. PIMT was added last to initiate the methylation reaction. Next, the reaction was mixed 1:1 (volume) with ^{14}N hydrazine (2 M, pH 8) and incubated at 37 °C for 17 h. A 20 μL aliquot of the sample was desalted by Zip Tip (C18) and eluted with 10 μL of 50% water, 50% CH_3CN, and 0.05% TFA, pH 1.5−2.0. The eluted peptide solution was then mixed 1:1 with 5 mM dansyl chloride in CH_3CN (prepared daily), and the mixture was incubated at 37 °C for 14 h. We found all dansyl chloride was consumed at the end of the reaction by either hydrolysis or reaction with hydrazides. Dansylation of the negative control, the methylation reaction without hydrazine treatment, was performed identically to that of the treatment. The dansylation reactions were then directly mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix at 1:1 ratio and spotted for MALDI-MS analysis.

MALDI-MS. MALDI-MS spectra were obtained using a Voyager DE-RP MALDI-TOF, 4700 or 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Framingham, MA). MALDI instruments were calibrated daily using external calibration mixtures contained in the Sequazyme peptide mass standards kit (P2-3143-00) purchased from Applied Biosystems. Calibration mixture 1 containing des-Arg^1-braykinin, angiotensin I, Glu^1-fibrinopeptide B, and neurotensin was used for analytes within the mass range of 904−1672 Da. Calibration mixture 2 containing angiotensin I, ACTH (1−17 clip), ACTH (18−39 clip), and ACTH (7−38 clip) was used for analytes within with the range of 1297.56 to 5734.6 Da. Samples were desalted prior to MALDI analysis using C18 standard bed Zip Tips purchased from Millipore (Bedford, MA) unless otherwise noted. The samples were then cocrystallized with a saturated solution of CHCA in 50% water, 50% CH_3CN, and 0.05% TFA for analysis. MALDI MS/MS predictions were performed using GPMAW 6.2.

FTICR Analysis. An LTQ-FTMS instrument (Thermo Fisher, Waltham, MA) equipped with a PicoView ESI source (New Objective, Woburn, MA) was coupled to an Ultimate 3000 nanoflow LC pump (Dionex, Mountain View, CA) to record spectra for aged calmodulin digest samples. Approximately 16 pmol samples were loaded for each run and separated using a self-packed reversed phase column (75 μm i.d. × 15 cm, Magic C18 resin, 3 μm particle size, 200 Å pore size, Michrom Bioresources, Auburn, CA). The flow rate was approximately 200 nL/min for both sample loading and separation. A shallow gradient elution, using 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B), was performed by starting at 2% mobile phase B and increasing to 40% mobile phase B over 35 min, then to 90% mobile phase B over 10 min, and finally a constant 90% B for 10 min. Before the next sample was injected, the C-18 column was equilibrated at 2% mobile phase B for 30 min. At least one blank run was used to minimize the carryover effect between two different injections. The ion transfer tube of the linear ion trap was held at 245 °C, and the ion spray voltage was at 2.0 kV. The mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. A full MS (m/z 400−1800) scan was acquired in the FTICR cell with the mass resolution of 100 000 at m/z 400 (target ion counts at 2×10^6 ions), followed by nine sequential data-dependent MS/MS scans, which isolated the top nine ions with highest intensity in the former MS scan. For the data-dependent mode, the dynamic exclusion was utilized with two repeat counts (repeat duration of 30 s, exclusion list 200, and exclusion duration of 30 s). The normalized collision energy was 28% for MS/MS scans.

LCQ Analysis. Additional LC-MS data was obtained from a Surveyor HPLC system (Thermo Fisher, Waltham, MA) coupled to an LCQ ion trap mass spectrometer (Thermo Fisher, Waltham, MA) equipped with a PicoView ESI source (New Objective, Woburn, MA). Approximately 10 pmol of sample was loaded on a self-packed reversed phase column (75 μm i.d. × 15 cm, Magic C18 resin, 3 μm particle size, 200 Å pore size, Michrom Bioresources, Auburn, CA) and eluted at 200 nL/min. A gradient of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B) was performed by starting at 1% mobile phase B and increasing to 10% mobile phase B over 5 min, then increasing to 40% mobile phase B over 30 min, and finally increasing to 80% B in 5 min. Before the next sample was injected, the C18 column was equilibrated and desalted at 1% mobile phase B for 40 min. The ion transfer tube of the linear ion trap was held at 250 °C, and the ion spray voltage was set at 2.3 kV. The mass spectrometer altered between a full MS scan (m/z 400−2000) and three sequential data-dependent MS/MS scans, which isolated the top three ions with highest intensity from the former MS scan. The normalized collision energy was 40% for

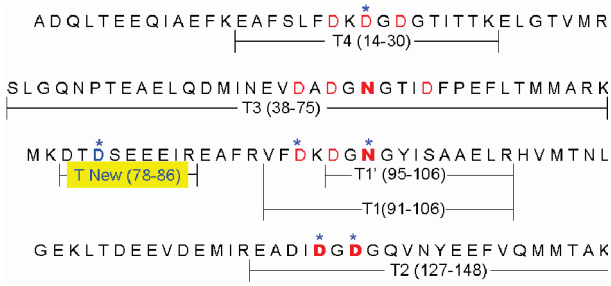


Figure 1. IsoAsp sites in aged calmodulin. Red denotes sites for aged protein (ref 25) with major sites bolded. Blue denotes sites identified in affinity-purified protein (ref 31). Asterisks denote hydrazide sites verified by sequence analysis by tandem MS from this work.

each of the MS/MS scans.

Data Processing. All the LC-MS/MS data was analyzed by using the Xcalibur 2.0 software (Thermo Fisher, Waltham, MA). FT-MS/MS spectra were extracted and searched against a protein database, containing bovine brain calmodulin protein sequence (see Figure 1), using SEQUEST algorithm. The variable modification was set for different amino acids before and after hydrazine modification. Briefly, for the ^{14}N reagent labeled sample: +15 Da on asparagine (Asn), +14 Da on aspartic acid (Asp), and +16 Da on methionine (Met); for ^{15}N reagent labeled sample: +17 Da on Asn, +16 Da on Asp, and +16 Da on Met, were employed to the database search. Specific tryptic cleavage was used with a mass tolerance of 1.5 amu for the monoisotopic precursor. The SEQUEST results were then filtered using the program Bioworks 3.2 (Thermo Fisher, Waltham, MA) requiring XCorr cutoffs of 1.9, 2.2, and 3.75 for +1, +2, and +3 charged peptides, respectively; a peptide probability cutoff of 0.01; and fully tryptic status of identified peptides. Peak areas of select peptides were extracted manually using QualBrowser software (Thermo Fisher, Waltham, MA) in both FT-MS and LCQ data. The mass tolerance was 1 amu for precursors in LCQ data.

RESULTS AND DISCUSSION

Analysis of Protein Isoaspartate Residues by Affinity Enrichment of Peptidyl Hydrazides. Affinity enrichment can greatly increase the sensitivity and selectivity of the chemical analysis of isoaspartate residues in complex mixtures of peptides and proteins. As shown in Scheme 2, aldehyde resins can be used to affinity enrich peptidyl hydrazides derived from isoaspartates, representing a significant advantage imparted by hydrazine labeling. Figure 2 shows spectra of calmodulin tryptic digest labeled with hydrazine before (top) and after (bottom) affinity enrichment. The efficiency of the affinity-enrichment process is obvious as only a handful of peaks remain after enrichment; the two major peaks are confirmed to be from isoaspartyl hydrazides. The peak at 1769.5 (highlighted in red) corresponds to the calmodulin T1 ^{14}N hydrazide modified at isoAsp97 (see Figure 1 for amino acid sequence), as determined by MALDI-TOF-TOF-MS/MS (data not shown). The peak at 1783.5 (highlighted in blue), which was undetectable previous to affinity enrichment, corresponds to the T1 peptide ^{14}N hydrazide modified at two sites, isoAsp95 and isoAsp97, as determined by MALDI-TOF-TOF-MS/MS, see Figure S2.1 in the Supporting Information. The modification positions for both the singly and doubly modified T1 peptides

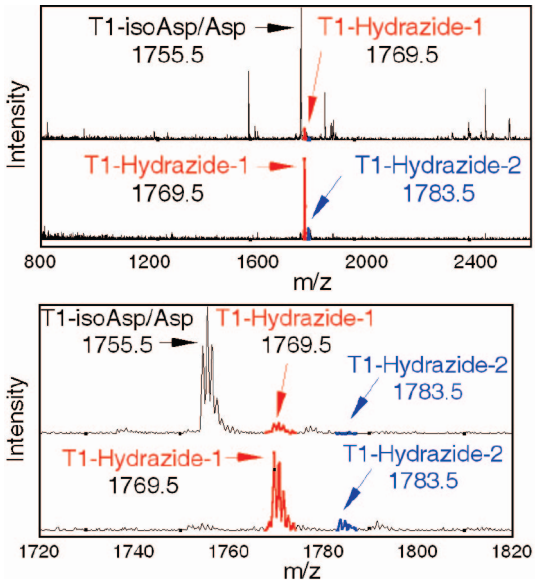
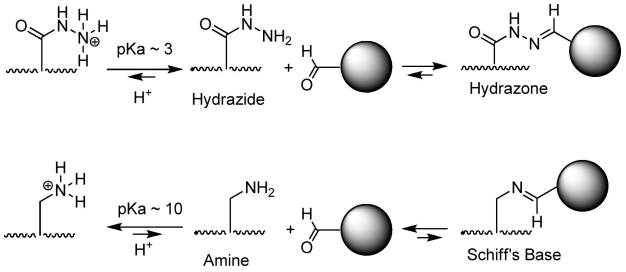


Figure 2. MALDI MS spectra of labeled calmodulin tryptic digest before (top) and after (bottom) affinity enrichment. The peaks colored in red and blue correspond to two isoaspartyl hydrazides.

Scheme 3. Selective Binding of Hydrazides against Amines to Aldehyde Resins at Mildly Acidic Conditions (pH 3−6)



agree with known isoaspartate forming sites in age-damaged calmodulin as determine by Aswad and co-workers.[25] In addition, two-dimensional solution NMR has been used to detect deamidation at Asn97, and the data suggest IsoAsp is formed (personal communication, Jennifer Laurence). As it can easily be seen, the affinity-enrichment method we have introduced here markedly reduces sample complexity and enriches low-abundance peptides, such as the doubly modified T1 peptide (peak at 1783.86) to a detectable quantity. Similar results were obtained with other peptidyl hydrazides (such as LHRH hydrazide, data not shown). Hence, our results have demonstrated the utility of our methods for the detection of peptide hydrazides in complex mixtures.

This high degree of selectivity in binding hydrazide against free amine is achieved under mildly acidic conditions (e.g., pH 5) where the majority of hydrazides ($pK_a \sim 3$) remain neutral, while the majority of amines ($pK_a \sim 9$) are protonated, as illustrated in Scheme 3. As such, the hydrazides are retained on the resin as hydrazones, while amines are washed away with pH 5 buffer. Elution of hydrazides can be accomplished under strongly acidic condition by shifting the equilibrium of the reaction to favor dissociated protonated hydrazides. However, we chose a milder alternative to avoid sample and resin degradation, where elution of hydrazides was accomplished using acetohydrazide solutions

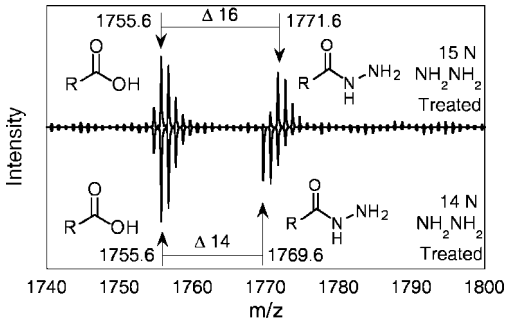


Figure 3. MALDI MS spectra of calmodulin labeled with ^{15}N hydrazine (up trace) and ^{14}N hydrazine (down trace).

at pH 3. In terms of recovery yields (50−60%) and selectivity, the milder elution conditions were comparable to more strongly acidic conditions.

Methylation and Hydrazinolysis. The specificity of the PIMT-catalyzed methylation of isoAsp residues has been extensively investigated, indicating that most isoAsp residues in peptides are recognized with high affinity.[26] Of course, protein tertiary structures are likely to affect the accessibility of the isoAsp residues, and thus we chose to label the peptide digests instead of the intact proteins. As discussed above, deamidation of Asn is a spontaneous process and the rates depend on pH, temperature, and other factors. Hence, care should be taken to minimize this process during sample handing.[27] For example, we performed the tryptic digestion in a short period of time (about 2 h) and close to neutral pH. Of course, control experiments, such as the time-dependent aging studies reported herein, should be conducted to assess the relative stability of each Asn or Asp residue.

In the pivotal step, isoaspartyl methyl ester and succinimide react with hydrazines or hydroxylamines, which are effective in trapping enzyme acyl intermediates and cleaving protein thioesters.[28,29] Isoaspartate-containing peptides and the well-characterized aged calmodulin protein have been successfully labeled with yields up to 50%, when treated with 1 M hydrazine, pH 7.4−8.1, at 37 °C from 1 to 8 h. However, subsequent analyses suggested that shorter treatment is sufficient for labeling. For example, β-delta sleep-inducing peptide (DSIP, Trp-Ala-Gly-Gly-isoAsp-Ala-Ser-Gly-Glu) isoaspartyl methyl ester reacts with 1 M hydrazine at pH 8 to completion within 1 h at 37 °C with a 50% hydrazide yield (see Figures S3.1.1−3 in the Supporting Information).

Analysis of Hydrazides. Compared to the labile methyl esters and succinimides, hydrazides are stable under various conditions used for LC and MS. Figure 3 shows spectra of the aged calmodulin T1 tryptic peptide (see Figure 1 for sequence information),[25] which is known to contain an isoAsp residue, labeled with ^{14}N or ^{15}N hydrazine. The strong signals for the ^{14}N and ^{15}N hydrazides are easily distinguished from those of the unmodified Asp and isoAsp peptides. The stable isotope labeling strategy assists with peptidyl hydrazide identification in complex mixtures by flagging hydrazides with a $\Delta 2$ Da mass shift, easily visualized by the "head to tail" analysis as shown in Figure 3. Of course, these mass shifts can be readily detected by a pattern recognition algorithm that is widely available in proteomic analysis.

Peptide Hydrazide Identification via LC-FTICR-MS Analysis. We also identified several other known calmodulin isoAsp peptides and determined the precise location of isoAsp in all of them. Table 1 shows hydrazide peptides from tryptic digested calmodulin (crude, aged 25 days) treated with ^{14}N hydrazines identified by LC-FTICR-MS and MS/MS analysis (see Table S3.2.1 in Supporting Information for ^{15}N hydrazides). The mass accuracy observed was in the typical range for LC-FTICR-MS analysis.[30] Hydrazide sites were identified as Asn97 in T1 (91−106), Asn97 in T1′(95−106, the proteolytic cleavage product of T1), Asp131 and Asp133 in T2 (127−145), Asp22 in T4 (14−30), based on precursor ion mass accuracy and MS/MS spectra (see Figures S3.2.1 to S3.2.7 in Supporting Information). These sites agree with isoAsp sites reported by Aswad's group.[25] The T3 hydrazide was not observed, possibly due to the weak ionization efficiency of the peptide (pI 3.58) in the positive mode. In addition, we identified an isoAsp peptide that was not reported by Aswad's group, referred to as T-new (78−86), modified at Asp80 as determined by MS/MS analysis (see Figures S3.2.8−9 in Supporting Information). This T-new peptide, however, was shown to be a major methylation site in native calmodulin by Ota and Clarke.[31] They narrowed the putative modification site to either Asp78 or Asp80, but were unable to pin down the precise amino acid residue at that time. Table 1 shows the relative abundances of the T peptide hydrazides, which were estimated based on abundances relative to Asp/isoAsp and Asn peaks. We observed relative abundances as high as 23% for the T1 hydrazide. Additionally, we found that the abundances of the T hydrazide peptides relative to one another follow the order for methyl accepting ability (isoAsp content) observed by Aswad and co-workers; hydrazide abundances were in the following order, T1, T2 and T4. The T-new peptide hydrazide had the lowest abundance (0.3% hydrazide peak). This may be the reason why it was not observed in Aswad's studies. Ota and Clarke used radioactivity labeling, which was extremely sensitive, and thus were able to detect this peptide. As can readily be seen in Table 1 and an extracted ion chromatogram (see Figure S3.2.10 in the Supporting Information) the retention time of the hydrazide species is close to those of the corresponding isoAsp, Asp, and Asn species. The hydrazide peak tails, potentially due the presence of multiple protonation states of the hydrazide (pK_a 3) during elution at pH 2 (0.1% formic acid in eluent) and the presence of isoaspartyl hydrazide and aspartyl hydrazide. The retention time of the hydrazide relative to isoAsp and Asp may serve as an additional constraint in the identification of hydrazide species, assisting in ruling out false positives that may arise in extracted ion chromatograms. In addition to LC MS/MS, peptides were identified by MALDI MS as well (see Figures S3.2.1−S3.2.16 in the Supporting Information).

Hydrazinolysis with Substituted Hydrazines and Hydroxylamine. In addition, isoAsp peptides were labeled with various substituted hydrazines (e.g., methyl hydrazine, *n*-butyl hydrazine, and propargyl hydrazine) as shown in Scheme 1 (see Section S3.4

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Table 1. Identification and Relative Abundance of Hydrazine-Modified Peptides in Calmodulin Tryptic Digests by LC-FTICR-MS and MS/MS[a]

aged calmodulin tryptic fragment		$^{14}NH_2{}^{14}NH_2$ treated digest				
		calculated $[M + H]^+$	observed $[M + H]^+$	MA (ppm)	relative abundance	RT (min)
T1 (91−106)	VFDKDG**N**GYISAAELR	1754.8707	1754.8777	+4.0	12	24.7
	VFDKDG**X**GYISAAELR	1755.8547	1755.8572	+1.4	66	25.1
	VFDKDG**Z**GYISAAELR	1769.8816	1769.8900	+4.7	**22**	25.5
T1′ (95−106)	DG**N**GYISAAELR	1265.6120	1265.6112	−0.6	25	23.9
	DG**X**GYISAAELR	1266.5960	1266.6014	+4.3	67	24.2
	DG**Z**GYISAAELR	1280.6229	1280.6274	+3.5	**8**	23.6
T2 (127−145)	EADIDG**X**GQVNYEEFVQMM	2221.8899	2221.8931	+1.4	86	27.7
	EADIDG**Z**GQVNYEEFVQMM	2235.9168	2235.9280	+5.0	**14**	27.8
T3 (38−74)	SLGQNPTEAELQDMINEVDADG**N**GTIDFPEFLTMMR	4117.8310	not detected			
	SLGQNPTEAELQDMINEVDADG**X**GTIDFPEFLTMMAR	4118.8150				
	SLGQNPTEAELQDMINEVDADG**Z**GTIDFPEFLTMMAR	4132.8419				
T4 (14−30)	EAFSLFDK**X**GDGTITTK	1844.8912	1844.8966	+2.9	99	27.1
	EAFSLFDK**Z**GDGTITTK	1858.8981	1858.9003	+1.2	**1**	27.0
T-new	DT**X**SEEEIR	1093.4644	1093.4612	−3.0	99.7	16.2
	DT**Z**SEEEIR	1107.4913	1107.4992	+7.1	**0.3**	16.7

[a] MA stands for mass accuracy and was calculated from (observed value − calculated value)/calculated value in units of ppm. X in the peptide sequence represents isoAsp or Asp residues, and Z represents the hydrazide. Underlined M residues represent the oxidized methionine residues. The relative abundance was estimated by the percentage of specified peptide in the sum of peak area of all peptide forms from the extracted ion chromatogram (XIC). Peak areas of hydrazide peptides were extracted and determined with a mass accuracy of ± 10 ppm.

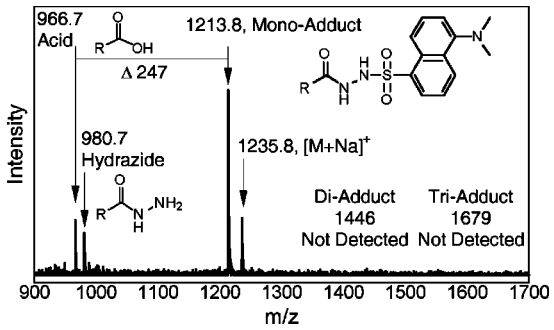

Figure 4. MALDI spectrum of dansylated KASA-isoD-LAKY hydrazide.

in the Supporting Information for experimental data). Labeling with propargyl hydrazine opens opportunities to further derivatize hydrazides with azide-containing tags using click chemistry.[32] Similar to hydrazine, we found that other nucleophiles, such as hydroxylamines, could also label isoAsp, as recently reported by Zhu and Aswad.[33]

Dansylation of Peptidyl Hydrazides. Spectrometric and fluorescent detection remain attractive alternatives to MS, as the required instruments are more widely available and offer direct identification and quantification. We found that the peptidyl hydrazides can be selectively modified by fluorogenic dansyl chloride, under mild acidic conditions similar to affinity binding (Scheme 3), with high yield and no detectable modification of the peptide's amines (N-terminus and two lysines), as illustrated in Figure 4. The peak at 1213.80 corresponds to dansylated peptide hydrazide and displays a mass shift of 247 Da from the isoAsp peak at 966.69, a mass shift of 233 Da for the hydrazide peak at 980.72. The dansylated peptide was sequenced by MALDI-TOF-TOF-MS/MS, and the modification site was confirmed to be isoAsp 5 (see Figure S4.2 in the Supporting Information). Moreover, dansylation was not detected in the negative control (see Figure S4.1 in the Supporting Information). One of the additional experiments was dansylation of the peptidyl hydrazide (10 μM) in the presence of methionine (10 mM), a ratio of 1:1000, in 300 mM sodium citrate at pH 3. Reaction products were analyzed by HPLC and monitored by UV−vis detection. Dansylated methionine was not detectable under these conditions (data not shown), indicating a selectivity of greater than 1:10000 in favor of hydrazides against amines. The observed high selectivity of hydrazides vs amines toward electrophiles, such as dansyl chloride, was achieved under the similar mildly acidic conditions used for the selective reaction of hydrazide with aldehyde. Given the selectivity of the dansylation reaction, we foresee using this labeling strategy to introduce the sulfonyl fluorophore/chromophore for UV−vis or fluorescence detection coupled with HPLC or two-dimensional gel electrophoresis to directly detect and quantify isoAsp peptides without MS.

CONCLUSIONS

In conclusion, we have developed a specific and flexible method for isoaspartate analysis that offers significant advantages over and is also complimentary to the existing methods. First, the peptidyl hydrazides generated from isoaspartates, in contrast to the labile methyl esters, are stable and can be readily analyzed by standard LC and MS. Second, the chemical trapping step allows the introduction of several tagging strategies for product identification and direct quantification. Third and most significantly, the peptidyl hydrazides can be affinity enriched markedly with high selectivity and yields, thus making it feasible to interrogate

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complex systems. Of course, the isoaspartyl residues identified by our method can be independently verified by other methods, such as electron capture dissociation (ECD) MS developed by O'Connor and co-workers.[9–11] Altogether, our method reported herein will be of great utility to biological studies, proteomic research, and pharmaceutical development.

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SUPPORTING INFORMATION AVAILABLE

Experimental procedures and characterization of modified peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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