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Identification and quantitation of tetrapeptide deamidation products by mass spectrometry*

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Abstract: A method to quantify asparagine (Asn), aspartate (Asp) and isoaspartate (isoAsp) residues in small peptides by fast atom bombardment mass spectrometry (FAB-MS) was developed. Discrimination of isoAsp from Asp residues was accomplished by selective derivatization of isoAsp residues in acetic anhydride, D_2O and pyridine. Deuteration occurred at any carbon adjacent to a free α-carboxyl group, through a transient oxazalone intermediate, allowing the isoAsp side chain and the C-terminus to incorporate deuterium. Thus, isoAsp-containing peptides incorporate one more deuterium than peptides with Asp and two more than Asn peptides. FAB CID-MS spectra of the Asn tetrapeptide, Thr-Asn-Ser-Tyr, were used to confirm the position of deuteration to the C-terminal residue. FAB and FAB CID-MS spectra demonstrated that the 1 amu shift in mass was not caused by derivatization induced deamidation of the Asn residue. FAB-MS spectra of deuterated peptide standards and mixtures containing deamidation products were obtained over the molecular ion region and deconvoluted using non-deuterated control spectra. Deuterium incorporation values for the Asn, Asp and iosAsp containing peptide standards were 80% mono-deuterated peptide, 95% mono-deuterated peptide and 63% di-deuterated peptide, respectively. IsoAsp to Asp ratios in an unknown mixture were obtained by a least-squares minimization of the difference between the unknown deuterated mixture and the isotopic envelopes from the deuterated standards. The mixture was found to contain 85% isoAsp peptide by FAB-MS, which agreed well with 81% isoAsp peptide when assayed by reversed-phase LC.

Keywords: *Peptide deamidation; selective deuteration; fast atom bombardment mass spectrometry; reversed-phase LC.*

Introduction

Recombinant DNA technology has made possible the large-scale production of pharmaceutically active peptides and proteins. However, stabilization of these macromolecular structures and the retention of potency have posed difficult problems. In particular, deamidation of asparagine (Asn) residues to aspartate (Asp) and iso-aspartate (isoAsp) residues is a major chemical degradation pathway [1]. Asn residues undergo a net hydrolysis reaction, proceeding through a cyclic imide intermediate at neutral and basic pH, and via direct hydrolysis of the amide side chain at acidic pH (Fig. 1) [1–5].

These degradation products are often difficult to separate and identify due to the relatively small change, located within one residue, in the overall characteristics of the polypeptide structure. For many proteins and peptides successful separations have been achieved using reversed-phase LC. These separations are usually accomplished on relatively small peptides, whereas separation of higher molecular weight peptides and proteins is problematic. Furthermore, not all peptide sequences exhibit altered retention characteristics upon deamidation. For example, adrenocorticotropic hormone (ACTH) deamidation products could not be resolved using reversed-phase LC, cation-exchange LC or isoelectric focusing [6]. In addition, identification and quantitation of isomeric degradation products is difficult. Standard identification methods include protein sequencing and enzymatic methods. Sequencing by Edman degradation will only cleave Asp residues, but cannot accommodate isoAsp residues due to the extra methylene incorporated into the peptide back-

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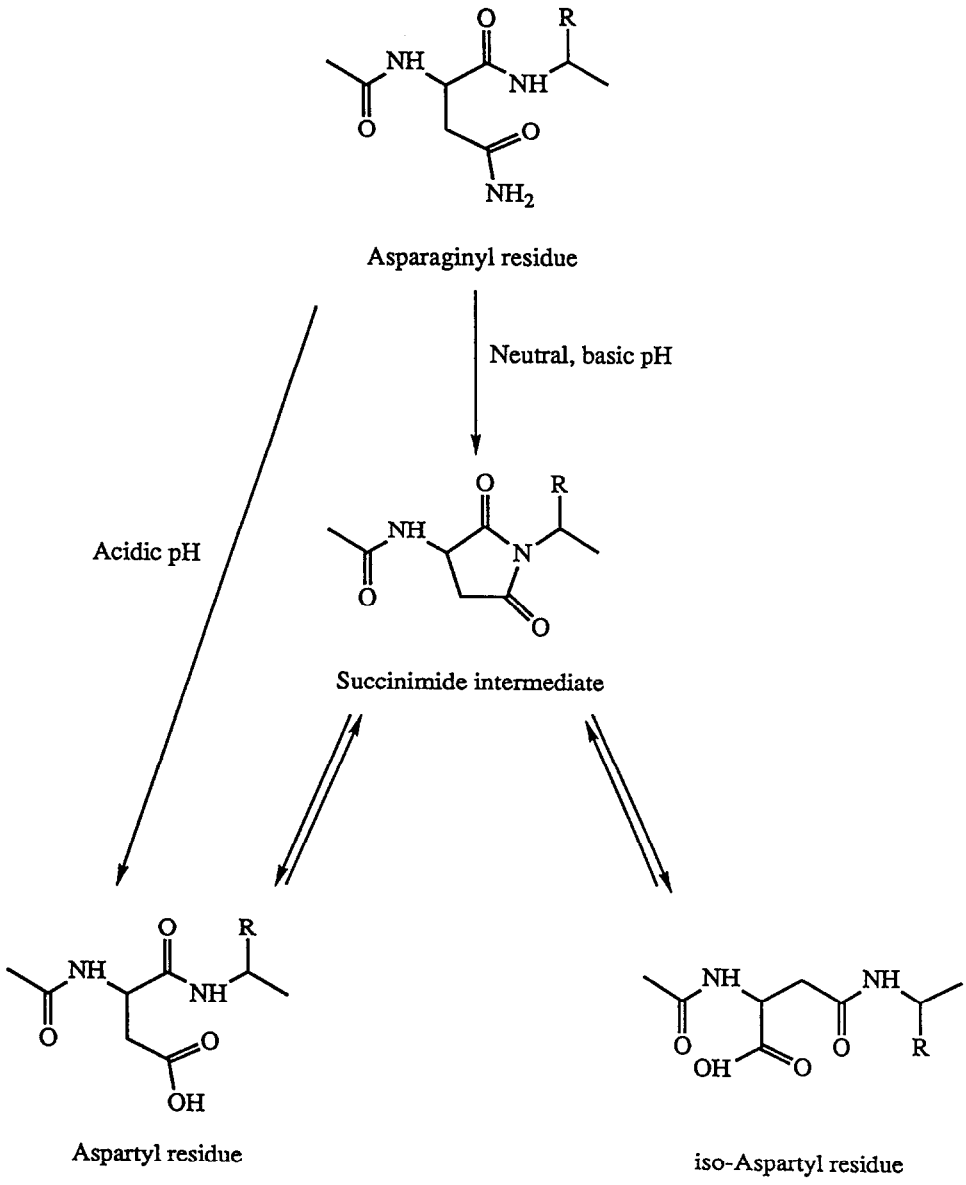


Figure 1
Mechanistic scheme for the deamidation of asparagine residues. At neutral and basic pH, the reaction proceeds through a cyclic imide intermediate formed by intramolecular attack of the backbone nitrogen on the Asn side-chain carbonyl. Spontaneous hydrolysis yields either an α-(Asp) or β-carboxy (isoAsp) linked aspartate product. At acidic pH, the reaction involves direct hydrolysis of the amide side-chain to form only the Asp isomer.

bone [7]. Enzymatic digest with Protease V8 from *Staphylococcus aureus* will only recognize Asp and subsequently cleave on the C-terminal side of these residues [8, 9]. Again, Protease V8 cannot distinguish between Asn and isoAsp residues, and can cleave C-terminal to Glu residues. Lastly, protein carboxyl methyltransferase has been shown to quantitatively methylate isoAsp peptides, but does not methylate Asp or Asn-containing peptides [10–12].

Fast atom bombardment mass spectrometry (FAB-MS), combined with selective derivatization, is proposed as a third method for identifying and quantifying Asn, Asp and isoAsp residues, which does not require prior separation of the differing peptide species. The model tetrapeptide, Thr-Asn-Ser-Tyr (MH^+ = 484), rapidly deamidates to yield chromatographically separable isomeric products, Thr-Asp-Ser-Tyr and Thr-isoAsp-Ser-Tyr (MH^+ = 485). The tetrapeptide fragment corresponds

to residues 7–10 in human growth hormone releasing factor (hGRF), which has been shown to deamidate readily with loss of biological activity [13]. Development of an alternative identification method was pursued to identify the deamidation products of several related hGRF analogues where material was a limiting factor. Furthermore, this primary sequence is not exclusive to GRF analogues, but is also highly conserved in growth hormones [14]. Bovine growth hormone has been shown to deamidate readily at position 99, where residues 98–101 are Thr-Asn-Ser-Leu [14–16]. Therefore, a method by which the tetrapeptide fragment could be identified and quantified was of interest to two protein classes susceptible to rapid deamidation.

Experimental

Materials and reagents

Crude peptide was obtained from Peninsula Laboratories (Belmont, CA, USA) and purified on an Econosphere C-18 semi-preparatory column monitoring at 275 nm. A gradient of 1% min^{-1} from 10 to 20% B was used where mobile phase A was 0.1% trifluoroacetic acid in water (v/v) and mobile phase B was 0.1% trifluoroacetic acid in acetonitrile (v/v). Acetonitrile (HPLC grade), trifluoroacetic acid, ammonium bicarbonate and pyridine were purchased from Fisher Scientific (Fairlawn, NJ, USA). D_2O (99.996%) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA).

Instrumentation

Samples were reconstituted in 0.1% trifluoroacetic acid and applied to the probe in a thioglycerol–glycerol (2:1, v/v) matrix. Mass spectra were obtained on either a ZAB-HS (VG Analytical Ltd, Manchester, UK) interfaced with an 11/250 data system, or a JEOL SX102 (JEOL Ltd, Tokyo, Japan). ZAB-HS FAB-MS studies were performed with a xenon atom gun operated at 8 keV and 0.8 mA emission. Studies on mixtures of peptides were carried out by scanning over the molecular ion region. Data was acquired in continuum mode by accumulating 5–10 scans. Collision induced dissociation (CID) spectra were acquired on the ZAB-HS using the linked scan at constant B/E technique. Collision spectra were recorded in multichannel analyser (MCA) mode under data system control. An exponential down-scan over the range 950 to 5 amu was acquired at 10 s dec^{-1}. An average of six scans was acquired per sample. The collision energy was 8 keV (laboratory frame of reference). For CID spectra, precursor ion current was attenuated to 40% with helium in the first field free region gas cell. FAB-MS analysis on the JEOL SX102 was performed at 10 keV acceleration potential using a xenon atom gun operated at 6 keV energy and scanning from 100 to 1000 *m/z* at 10 s dec^{-1}. Both instruments were operated at 1000 resolution.

Peptide deamidation

Thr-Asn-Ser-Tyr was deamidated in 0.1 M NH_4HCO_3, pH 10.0 at 37°C for approximately 24 h. An isocratic LC mobile phase of acetonitrile–water–trifluoroacetic acid (7:93:0.1, v/v/v) was used to separate the deamidation products. Deamidation progress was monitored on a reversed-phase HPLC system consisting of an Econosphere C-18 (250 mm × 4.6 mm, 5 μm) column, two Shimadzu LC-6A pumps, a SIL-6A autosampler, a SCL-6A controller, a CR3A integrator and a SPD-6A UV detector. The autosampler was linked to a Brinkman Lauda RM 20 water-bath for temperature control at 37°C.

Peptide derivatization

Peptide (100–200 nmol) was reconstituted in 250 μl acetic anhydride and 1 ml pyridine for 30 min, at room temperature. After 30 min, 500 μl D_2O was added to the solution and the reaction proceeded for an additional 2 h. The sample was then lyophilized to dryness. Two consecutive back-exchange steps were performed by reconstituting the peptide in 500 μl H_2O for 30 min and lyophilizing to dryness. Water was substituted for D_2O to provide controls for each sample.

Calculations

Data manipulations of isotopic envelopes were performed via least-squares minimization [17] using an adaptation of a matrix algebra method [18] or a Lotus spread sheet.

Results and Discussion

The selective deuteration of β-Asp (isoAsp) and γ-Glu residues for identification purposes has been reported [19–21]. The method involves formylation of the N-terminus, deuteration with acetic anhydride, D_2O and pyridine,

and analysis by FAB-MS [20]. It was found that simplification of the preceding method by eliminating the formylation step allowed us to quantitate deamidation product mixtures. The resulting peptide was acetylated at the N-terminus and at all residue side chains except position 2. The C-terminus and the β-carboxy-linked isomer isoAsp can be preferentially deuterated, over Asp residues, at the carbon adjacent to the free α-carboxyl group, through a transient oxazalone intermediate (Fig. 2). The alpha proton becomes very acidic during ring formation and readily exchanges with deuterium. Thus, the deuterated isoAsp containing peptide will result in a peptide one mass unit larger than the Asp peptide and two mass

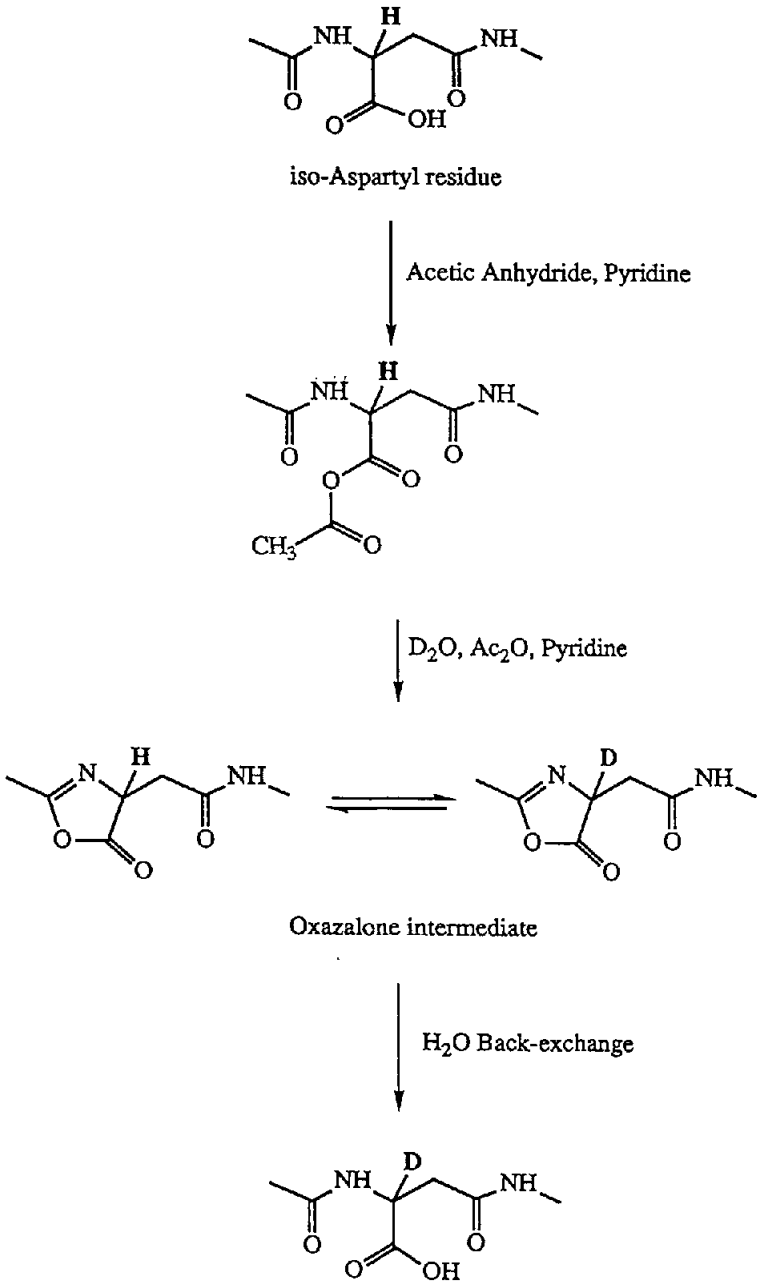
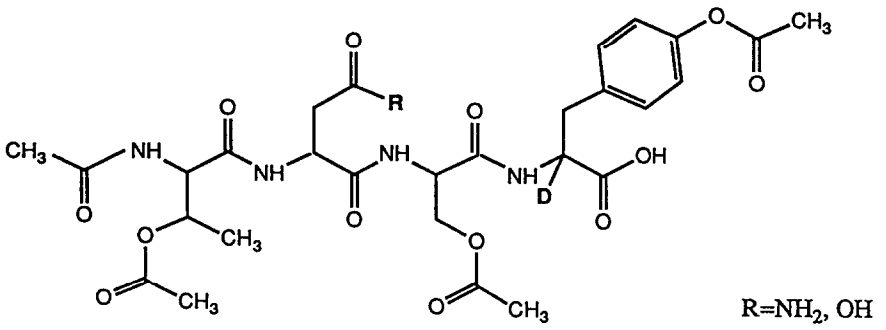
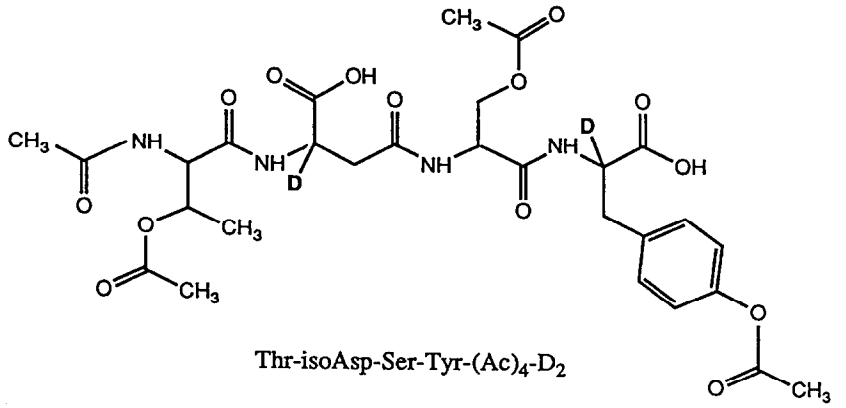


Figure 2
Deuteration of C-terminal carboxylic acids and isoAsp residues via a transient oxazalone intermediate. Preferential deuteration occurs at the C-terminus and isoAsp residues, which can readily form the five-membered ring intermediate, but not at Asp or Asn residues.



Thr-Asx-Ser-Tyr-$(Ac)_4$-D_1



Thr-isoAsp-Ser-Tyr-$(Ac)_4$-D_2

Figure 3
Tetrapeptide structure and monoisotopic mass for derivatized Asn-(MH^+ = 653), Asp-(MH^+ = 654) and isoAsp-(MH^+ = 655) containing peptides. The Asp- and Asn-containing peptides incorporate one deuterium while isoAsp deuterates at two sites.

units larger than the Asn peptide, making the isobaric Asp and isoAsp isomers differentiable by mass spectrometry (Fig. 3).

Prior to analysing deuterated peptide mixtures, discrimination studies on peptides differing by 1 amu were performed on mixtures of Thr-Asn-Ser-Tyr (MH^+ = 484) and Thr-Asp-Ser-Tyr/Thr-isoAsp-Ser-Tyr (MH^+ = 485) to demonstrate that deamidation content could be quantified down to 5% Asn or Asp using FAB-MS generated molecular ion clusters. The values in Table 1 were obtained by monitoring deamidation of Thr-Asn-Ser-Tyr by reversed-phase LC (Fig. 4) and collecting samples for FAB-MS at various time points. Without derivatization, Asp- and isoAsp-containing peptides are indistinguishable by MS, therefore LC peak areas for the Asp- and isoAsp-containing peptides were combined and compared to the remaining Asn-containing peptide. The per cent deamidated material, as calculated from LC peak areas, was then compared to that obtained by deconvoluting the molecular envelope observed from FAB-MS. Deconvolution was achieved by obtaining isotopic envelopes for the mixed peptide molecular ion region and calculating the contributions to that envelope from standard isotopic envelopes of the Asn and deamidated peptides. Values from LC and FAB-MS agreed well. The difference between values from each method was no more than 5%, where relative precision was no more than ±0.5% for duplicate LC injections and triplicate FAB stage loadings. Additional mixing studies on synthetic standards of Thr-Asn-Ser-Tyr and Thr-Asp-Ser-Tyr showed that deamidation content could be quantified down to 1% Asp, indicat-

Table 1
Comparison of LC and MS for the analysis of peptide deamidation·

% Deamidation by HPLC	% Deamidation by MS*
0.0	0.2 ± 0.1
10.4 ± 0.7	7.9 ± 0.3
29.6 ± 0.8	26.5 ± 0.4
57.6 ± 0.3	55.5 ± 0.7
73.4 ± 0.8	68.1 ± 1.5

Mixing study performed on Thr-Asn-Ser-Tyr- and Thr-Asp-Ser-Tyr-containing samples. Comparison of quantitation by LC peak area and MS showed that the FAB molecular ion envelope could be used to quantitate mixtures with molecular ions 1 amu apart.

*MS ions clusters were corrected with a standard Asn peptide isotopic cluster.

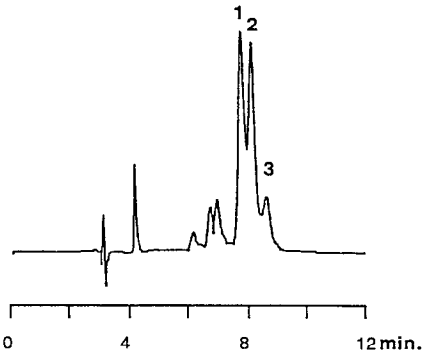


Figure 4
Reversed-phase LC chromatogram showing a separation of the tetrapeptide deamidation products where (1) indicates the isoAsp-containing peak, (2) indicates Asn peptide, and (3) indicates the Asp-containing peptide.

ing the sensitivity of the MS method when used independently.

Subsequently, a synthetic sample of Thr-Asn-Ser-Tyr was derivatized and characterized by FAB-MS and FAB CID-MS experiments to determine the extent and position of deuterium incorporation. The resulting molecular envelope was expected to shift by 1 amu as compared to the control sample. Quantitation of molecular abundances is potentially complicated by incomplete deuteration of sites, incomplete back-exchange and the natural abundance of isotopic variants, primarily ^{13}C. To identify these effects, the extent of deuteration was monitored in samples at 2, 3, 6 and 24 h with no change in extent of deuteration. Mass spectra on these samples were also collected over time to ascertain if further back-exchange took place in the FAB matrix. The isotopic envelopes were not seen to shift during mulitiple scans, day to day, or when a third back-exchange was performed on the same sample. Isotope contributions due to ^{13}C are randomly distributed within the peptide. The deuterated and non-deuterated control have the same random incorporation of ^{13}C, therefore, natural abundances of isotopes was accounted for when the water controls were used as the standard envelope during deconvolution. Deuteration could not be successfully driven to 100% completion, but was reproducible at 80% ± 3% for Thr-Asn-Ser-Tyr-$(Ac)_4$-D_1. Thus, the observed incomplete deuteration was due to the derivatization method.

The position of deuterium incorporation was then determined from FAB CID-MS experiments. The CID spectrum of a peptide show a series of sequence specific ions that have been labelled as A, B, C″ when originating from the N-terminus, and X, Y″, Z when attached to the C-terminus. The letter codes correspond to specific peptide backbone bond cleavages [22]. In this study, CID spectra were acquired using the linked scan at constant B/E technique with helium in the first free region collision cell. A CID spectrum from the parent ion of the non-derivatized peptide, Thr-Asn-Ser-Tyr, was acquired to determine the predominant fragmentation series. Strong series of Y″ and B ions were observed for the length of the non-derivatized peptide. Secondly, a CID spectrum from the derivatized peptide parent ion, Thr-Asn-Ser-Tyr-$(Ac)_4$-D_1, was obtained to determine the deuteration site specificity. C-terminal sequence ions, X_2, Y_1'', Y_2'' and Y_3'', showed a 1 amu shift in the deuterated sample while the N-terminal sequence ions, A_2, B_2 and B_3, did not shift, localizing the position of the deuteration site to the C-terminal residue. Sequence specific ions showed no detectable ion current corresponding to an Asn to Asp degradation, indicating that the derivatization procedure did not cause further deamidation. For example, there was no $B_2 + 1$ or $Y_3'' + 1$ signal detected. Therefore, the CID spectra demonstrated that the deuteration procedure was site specific to the C-terminus and did not cause additional deamidation.

The degree of deuteration for the Asn-, Asp- and isoAsp-containing peptides was then explored to determine if incorporation was consistent for all peptide standards. Synthetic standards of Thr-Asn-Ser-Tyr and Thr-Asp-Ser-Tyr were derivatized in order to determine if C-terminal deuterium incorporation went to

the same per cent completion for each analogue. The deuterated molecular ion envelopes were deconvoluted by correcting with the isotopic envelope from the water control. Figure 5 shows the molecular envelopes for the water controls and the deuterated samples for the pure peptide analogues. The Asn-containing peptide deuterated to 80% ± 3% [Fig. 5(A)] while the Asp-containing peptide deuterated repro-

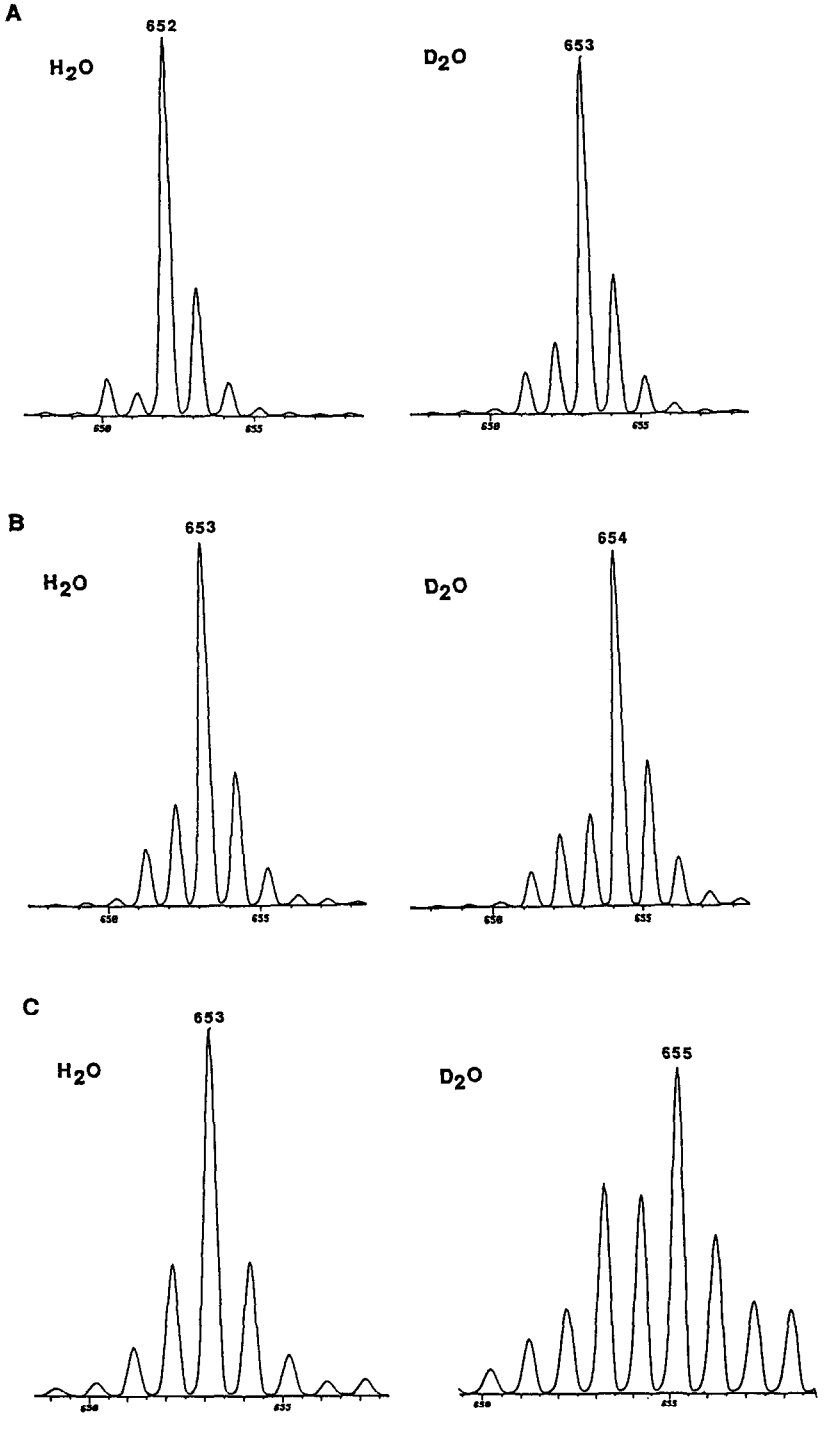


Figure 5
FAB-MS scans of tetrapeptide where (A) shows the H_2O control and the D_2O sample for Thr-Asn-Ser-Tyr-$(Ac)_4$ (MH^+ = 652) and Thr-Asn-Ser-Tyr-$(Ac)_4$-D_1 (MH^+ = 653), respectively. (B) and (C) show the water controls and the deuterated samples for Thr-Asp-Ser-Tyr-$(Ac)_4$-D_1 (MH^+ = 654) and Thr-isoAsp-Ser-Tyr-$(Ac)_4$-D_2 (MH^+ = 655).

ducibly to 95% ± 1% completion [Fig. 5(B)]. The variance in extent of deuteration indicated that the process may be sequence specific. Again, sequence ions in the FAB spectrum of the Asp-containing peptide indicated deuterium incorporation exclusively at the C-terminus. Thr-isoAsp-Ser-Tyr was then isolated by LC, deuterated and analysed by FAB-MS, where a 2 amu shift in mass over the control was expected [Fig. 5(C)]. Deconvolution of this envelope, using the H_2O control isotopic envelope, indicated 36% non-deuterated material and 63% of a di-deutero species, where the standard deviation was assumed to be similar to the Asn-containing peptide samples. Less than 1% of the cluster was attributed to a mono-deutero species.

A tetrapeptide deamidation mixture was then assayed by FAB-MS and reversed-phase LC for independent verification. Thr-Asn-Ser-Tyr is separable from its deamidation products by LC (Fig. 4). A sample of Thr-Asn-Ser-Tyr was deamidated to completion and assayed by LC, the peak areas indicated 81% Thr-isoAsp-Ser-Tyr and 19% Thr-Asp-Ser-Tyr. The LC peak areas were then compared with the results of the sample when derivatized and assayed by FAB-MS (Fig. 6). A least-squares minimization of the unknown mixture envelope (Fig. 6) and a generated envelope from the standard clusters (Fig. 5) was performed to determine the peptide mixture composition. Isotopic envelopes for the derivatized Asn, Asp and isoAsp standards, and the ion abundances for the unknown deamidated and deuterated mixture were entered into the spreadsheet. A starting ratio of Asn:Asp:isoAsp content was entered and the residuals from the generated cluster were compared to those calculated for the unknown mixture molecular envelope. The product ratio was altered until a minimum was determined, indicating the best fit, and gave 85% Thr-isoAsp-Ser-Tyr and 15% Thr-Asp-Ser-Tyr. This was in good agreement with the LC values (Table 1), considering that LC baseline separation of the three species was not achieved.

This method has been demonstrated to be useful for identification of degradation products as well as for quantitation purposes. Absolute quantitation has been shown to require standards of all the peptide variants. The method would most likely be useful in cases where the only available standard would be the starting material. Some assumptions about extent of deuteration would have to be made which require further study on the variability of peptide deuterium incorporation. Independent use of FAB-MS was shown to detect Asp-containing peptide content at 1%. The ability to detect 1% change in deamidation content suggests that this method could be

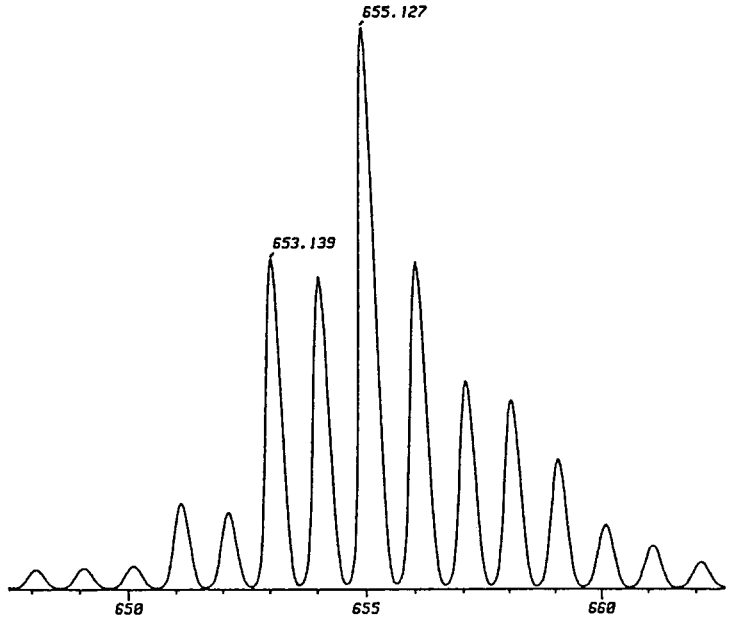


Figure 6
Molecular ion envelope for the deuterated unknown peptide mixture. Deconvolution of the isotopic envelope gave 85% isoAsp- and 15% Asp-containing peptide.

used to follow the initial stages of peptide deamidation very accurately. In conclusion, mass spectrometry provides a viable alternative to identification and quantification of deamidation products. It is, therefore, most applicable to small peptide systems that cannot be separated, identified or quantified by conventional means.

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