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Toward proteome-scale identification and quantification of isoaspartyl residues in biological samples

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Abstract

Deamidation of asparaginyl and isomerization of aspartyl residues in proteins produce a mixture of aspartyl and isoaspartyl residues, the latter being involved in protein aging and inactivation. Electron capture dissociation (ECD) combined with Fourier transform mass spectrometry (FT MS) are known to be able to distinguish the isoaspartyl peptides by unique fragments of $c_n\bullet + 58.0054$ ($C_2H_2O_2$) and $z_{l-n} - 56.9976$ (C_2HO_2), where *n* is the position of the aspartyl residue and *l* is the peptide length. In the present study, we tested the specificity of isoAsp detection using the accurate masses of the specific fragments. For this purpose we analyzed 32 whole and partial proteomes obtained from human cells as well as tissue samples and identified by ECD 466 isoaspartyl peptide candidates. Detailed inspection revealed that many of these candidates were unreliable. In order to increase the isoAsp detection specificity, additional criteria had to be used, e.g. adjacent c/z fragments, specific losses from the reduced species, and the shape of the chromatographic peak. Most stringent filtering of candidates yielded several cases where the presence of isoAsp was beyond doubt. Among the identified proteins with isoAsp, actin, heat shock cognate 71 kDa protein and pyruvate kinase have previously been identified as substrates for L-isoaspartyl methyltransferase, an important repair enzyme converting isoaspartyl to aspartyl. Quantification of relative isomerization degree was performed by the label-free approach. This is the first attempt to analyze the human isoaspartome in a high-throughput manner. The developed workflow allows for further enhancement of the detection rate of isoaspartyl residues in biological samples.

Keywords

proteomics; tandem mass spectrometry; electron capture dissociation; posttranslational modifications; Alzeimer's disease

Introduction

Deamidation of asparaginyl (Asn) residues and isomerization of aspartyl (Asp) residues are among the most prevalent posttranslational modifications (PTM) in proteins under physiological conditions. During deamidation reactions, the amide of asparaginyl residue is converted into carboxyl through a cyclic intermediate of succinimide, resulting in mass increase of 0.9840 Da (monoisotopic mass of Asn residue is 114.0429 Da and Asp residue 115.0269 Da) (Scheme 1). The hydrolysis of succinimide does not only generate aspartyl residues but also isoaspartyl (isoAsp) residues at a ratio around 1:3. [1,2] The prolongation of peptide backbone by the methylene group of isoaspartyl residues has been found to result in protein aggregation *in vitro* under physiological conditions. *In vivo*, iso-Asp has been

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associated to Alzheimer's disease (AD), aging and the loss of sight from cataract. [3–6] Protein deamidation is also the major source for degradation of protein pharmaceutical products. [7] Thus there is substantial need for reliable identification and characterization of deamidation and isomerization sites in proteins.

Generally, in spite of isotopic interference, it is straightforward to detect Asn deamidation by mass spectrometry (MS) because of the mass change of 0.9840 Da, and clear separation of Asn- from Asp-variants by liquid chromatography. Distinguishing aspartyl and isoaspartyl residues is much more difficult because they share the same molecular weight and have close physicochemical characteristics. Immunological methods, PIMT assays and Edman degradation have been reported to successfully differentiate the two isoforms. [3,8,9] HPLC can in many cases also separate Asp-peptides from isoAsp- variants, although the separation is smaller than for Asn/Asp peptide pairs. [3,10,11] However, there is still no efficient technical platform to identify isoaspartyl residues on the proteome scale. At the same time, proteomics studies are gaining strength and gradually becoming indispensable in biomedical research.

Mass spectrometry and tandem MS (MS/MS) have been useful analytical techniques in PTM analysis for years, but they are mostly used for PTMs that differ in mass (non-isomeric PTMs). Traditional collision-activated dissociation (CAD) can in certain cases distinguish aspartyl and isoaspartyl residues using the ratio of b/y fragments and/or the presence of b + H_2O peaks, but CAD-based techniques appear to be sequence dependent and lack specificity. [12] In electron capture dissociation (ECD) MS/MS, isoaspartyl residues, but not aspartyl residues, have been found to give specific c_n• + 58.0054 ($C_2H_2O_2$) and z_{l-n} − 56.9976 (C_2HO_2) fragments. These fragments are potential 'markers' for Asp isomerization; for simplicity, we will call them c ´+57 and z-57. [13] We have pioneered the use of ECD (together with CAD) in high-throughput analyses of whole proteomes for the purpose of enhanced peptide identification, [14] *de novo* sequencing [15] and unbiased determination of PTMs that differ in mass. [16] Furthermore, we have demonstrated the use of ECD for proteome-wide differentiation of constitutional isomers Ile and Leu. [17] Here we attempted proteome-scale identification of isoAsp residues due to Asn deamidation and Asp isomerization using the above specific fragments as indicators of isomerization. The main purpose of this study was to test the specificity of isoAsp detection using the accurate masses of the specific fragments. Such an effort is viewed as a first step towards understanding the biological role of the isoaspartome (proteome-wide isoAsp map). Another potential application of on-line isoAsp analysis is the routine quality test for protein pharmaceutical products. [12]

Experimental section

A 7 Tesla LTQ FT mass spectrometer (ThermoFisher Scientific, Bremen, Germany) was used to collect proteomics data from tryptic digests of whole cell lysates of human A431 cells and brain cell samples. The experimental procedure is described in detail in literature. [14,18] Briefly, each eluting peptide was ionized by electrospray and molecular ions were fragmented by ECD as well as CAD. CAD MS/MS spectra were used for peptide identification by Mascot search engine (Matrix Sciences, London, UK), while ECD confirmed the Mascot sequence assignment that received a Mascot score >20 using a home-written C++ program. The same program was used to search for the specific fragments of isoAsp: c_n• + 58.0054 ($C_2H_2O_2$) and z_{l-n} − 56.9976 (C_2HO_2) using their theoretical masses and the mass window of ±10 mDa.

Results and Discussion

Searching among 29 two-hour LC/MS runs of human A431 lysates and three 12.5-hour LC/MS runs of brain samples (211,445 CAD+ECD MS/MS queries) yielded 466 isoAsp candidates with either c_n´ + 57 or z_{l-n} − 57 fragment within given mass accuracy. Due to the possibility

of artifacts, additional confirmation was required before positive isoAsp identification could be pronounced. Often, these artifacts were due to spurious noise, and the candidate peaks were among the lowest-abundant peaks in the MS/MS spectrum. Sometimes, the expected fragment mass was within the isolation window of the precursor ion (±5 m/z units), and thus could be due to a co-isolated ion. In a few cases, the expected mass coincided within given mass accuracy with other fragments or their isotopic distributions. In all these cases, the corresponding candidate was discarded. All remaining candidates were manually investigated using the following criteria.

Chromatographic peak shape

It is known from literature that isoAsp isomerization proceeds via a metastable succinimide that has a typical life-time of 2–4 h at physiological pH and temperature. Upon spontaneous hydrolysis of succinimide, 70–85% of the product is isoAsp, the remainder being L-Asp and D-Asp. [19] Enzymes like PIMT (L-isoaspartyl methyltransferase) can change this ratio in favor of Asp, but their efficiency is limited (the average reparation rate is around 15–30% per cycle, and the overall repair efficiency could reach 85% or even higher). [20,21] Therefore, deamidation (as well as isomerization) leads to isomer mixture, where the isoAsp isomer is frequently not the most abundant component. Chromatographically, peptides with the isoAsp often elute earlier than their Asp-containing analogues. [12] Thus we were expecting to find for isoAsp candidates a complex shape of the chromatographic peak, with isoAsp peptide eluting (often, but not always) faster than the Asp counterpart.

Adjacent c and z fragments

The specific fragments $c_n' + 57$ and $z_{l-n} - 57$ are additional cleavages and they do not substitute the regular *c* and *z* ions around the isoAsp residue. Usually, the abundances of these adjacent fragments are higher than these of the specific fragments. Therefore, the presence of adjacent fragments strengthens the validity of the specific fragments.

Mass accuracy

Although specific fragments were detected with a ±10 mDa margin, higher accuracy (±5 ppm) was required if no other confirming feature was present.

Absence of specific Asp loss from reduced species

The loss of 60.0211 Da from the reduced species is a specific loss from the aspartyl residue, but it is not possible from the isoaspartyl residue. [22] Therefore, if only one Asp is found in the sequence, then the absence of such a loss is the absolute requirement for the isoAsp presence.

Isotopic peaks of the specific fragment

The presence of isotopic peaks distinguishes real ions from spurious noise peaks. However, specific isoAsp fragments frequently appear at low abundances, and thus sometimes exhibit no isotopic peaks.

CO_2 loss from c, z fragments

The losses of CO_2 are frequent from backbone fragments that have Asp or isoAsp as a terminal residue. The losses in case of Asp are often more abundant than from isoAsp. [23] Although this criterion is relatively “soft”, less abundant CO_2 losses compared to the unmodified peptide increase the confidence in isoAsp identity.

Complementary specific fragments

The presence of both $c_n' + 57$ and $z_{l-n} - 57$ fragments in the spectrum is a practical guarantee that at least one of these peaks is *not* due to spurious noise. However, such a double presence may still be due to a different kind of ions than the specific isoAsp fragments, as in the example below.

Same fragments in different spectra

Another confirmation for weak peaks of specific fragments is the presence of the same peak in other mass spectra of the same peptide, taken either during the same chromatographic peak, or in a different LC/MS analysis of the same or related sample.

Upon careful consideration of all 466 candidates, we have identified 219 cases when the specific fragment mass was supported by other evidence. In all cases, no 60.0211 Da loss from the reduced species was present when only one aspartyl residue was found in the peptide, 113 cases had more than one chromatographic peaks, 160 cases had more than one adjacent c/z fragment, 43 cases exhibited an isotopic distribution for the specific isoAsp fragment, 5 cases were found with complementary specific fragments, and 37 cases had a confirmation for the specific fragment mass either from another MS/MS scan in the same LC/MS run, or in a different LC/MS run for the same peptide. Among these “good candidates”, we selected eleven exceptionally convincing cases. Below we present several typical representatives of such cases (five cases of Asn deamidation and six cases of Asp isomerization) and discuss the pitfalls in validating the isoAsp identity.

Deamidation—Typical cases of Asn deamidation are summarized in Table 1. The chromatographic peaks and corresponding ECD spectrum of peptide LLY(N->isoD)NVSNFGR are shown in Figure 1. In the chromatogram, the three forms (native, deamidated, and isomerized) of the same sequence are well separated. Since there are MS/MS scans for each of the chromatographic peak, Mascot search confirms the identity for each of them. For the spectrum of isoAsp peptide, there is an isotopic distribution of the specific fragment with a loss of $z_8 - 57$, which proves that this is not a spurious noise. Among the neutral losses from the reduced species, no loss of 60.02 Da was present, which, if found, would invalidate the isoAsp assignment.

Another example of Asn deamidation is shown in Figure 2. The peptide LII(N->isoD)SLYK has been selected twice for MS/MS, as shown by the arrows on the chromatographic peaks. The last-eluting peak on the extracted ion chromatogram was due to an ion with the same m/z and charge state as the precursor ion, but this ion was not picked for MS/MS. Considering its shape and retention time, the last peak could be due to the Asp variant. In the ECD spectrum of the isoAsp peptide (lower panel), the specific fragment $z_5 - 57$ shows isotopic distribution, which obviously distinguishes it from the background noise. Also, there is a loss of ($\bullet C_3H_7 + NH_3$) from the reduced species $[M + 2H]^{+\bullet}$, confirming the presence of Leu in the sequence. [22] As for the u_4 ion, it is a loss of $\bullet C_3H_7$ radical from the Leu side chain in the z_4 ion. [24] Loss of $\bullet C_4H_8$ is also observed from z_4. [23]

Isomerization—Cases of Asp isomerization are summarized in Table 2. An example is shown in Figure 3. According to Mascot search, the two chromatographic peaks are due to identical sequences LDLAGR. In the ECD spectrum of the first-eluting minor component, an isoAsp specific fragment $z_5 - 57$ was found. In the spectrum of the Asp-variant, the loss of ($\bullet C_3H_7 + C_2H_4O_2$) from the reduced species (m/z 542.305) was attributed to a combined loss from the adjacent Leu and Asp residues. Such a combined loss has not been described in literature, but it is similar to known combined radical + molecule losses, e.g. the specific for Leu ($\bullet C_3H_7 + NH_3$) loss. [23] Since $C_2H_4O_2$ originates from the full Asp side chain, the

combined ($\bullet C_3H_7 + C_2H_4O_2$) loss cannot occur in the case of isoAsp. Consistent with that, the isoAsp ECD spectrum in Figure 3 does not contain this loss (the peak at m/z 541.309 does not seem to be related to that loss).

Another example, the peptide AGFAGD(D->isoD)APR with the isomerization of the second Asp, is shown in Figure 4. In the spectrum of both Asp and isoAsp peptides, there are losses of 60.02 ($C_2H_4O_2$) from the reduced species which could only occur from Asp but not isoAsp. However, there are two Asp residues in this peptide, which makes the loss of 60.02 reasonable.

Relevance to known substrates—Among all the proteins listed in Table 1 and Table 2, heat shock cognate, pyruvate kinase, and actin have been found to be substrates for PIMT (L-isoaspartyl methyltransferase) in mouse brain based on two dimensional PAGE MS experiments. [9] PIMT is an enzyme that could specifically convert isoaspartyl residues back to aspartyl residues, and is widely distributed in all mammalian tissues. Therefore, PIMT has been regarded as an essential repair enzyme acting against protein aging and damages caused by deamidation. [25,26] Although any aging protein could be a potential substrate for PIMT *in vivo*, our results here are consistent with the previous findings and also provide hints for the possible deamidation sites within the protein. Indeed, eight out of the eleven peptides that we found deamidated have been followed by either glycine (G), [27] serine(S), [28] or histidine (H). [29] This finding agrees with the previous suggestion that less bulky and more polar side chains of adjacent (especially from the C-terminal side) residues increase the deamidation probability. [30,31] The results again emphasize the importance of primary structure for protein deamidation, although the secondary and higher order structures could also influence the deamidation rate and product ratio of Asp and isoAsp. [32,33]

Confirmation from complementary fragments—Although it is rare to find both $c_n' + 57$ and $z_{l-n} - 57$ fragments present in the same spectrum (in ECD of dications, one of the complementary fragments is a neutral), we did observe at least one such case, peptide KVLGAFS(D->isoD)GLAHLDNLK. Figure 5 shows its ECD mass spectrum. As an additional confirmation, specific isoAsp fragments were also observed in ECD of the shorter form of the same peptide VLGAFS(D->isoD)GLAHLDNLK (the longer form was due to a cleavage site missed by trypsin).

Besides the above double confirmation, we have also observed a seemingly rare case of the double fragment mass overlap. When the peptide LDELRDEGK was detected (2+ ions at m/z 573.775), two specific fragments were found at m/z values corresponding to $c_5' + 57$ ion (sequence LDELR) at m/z 701.370 (LRDEGK) and $z_8 - 57$ ion (sequence DELRDEGK) at m/z 888.442. The same two ions were detected in an ECD spectrum of the same peptide taken half a minute later, which confirmed that the signal was not spurious. These data suggest *both* D residues in this peptide to be isoAsp. Since finding even a single specific fragment is rare, it would be natural to assume that the chance of double coincidence is so small that at least one of these two residues has indeed to be isoAsp. The problem is that the theoretical m/z values of these ions, 701.370230 and 888.442137, are very close to the theoretical m/z values of two conventional fragments, $z_6^{+\bullet}$ (LRDEGK) and $c_5^{+'}$ (LDELRDE), 701.370273 and 888.442151, respectively. The differences between the theoretical m/z values for the alternative assignments, 0.000043 and 0.000014 (61 and 16 ppb), are so small that for a long time will remain outside the capability of mass spectrometry to resolve. Note that the two fragments are *not* a complementary pair, and thus their masses are independent of each other. Such a case of double coincidence, however rare, reminds of the danger to draw conclusions solely based on accurately measured mass. While the overlap with common fragment types could be easily checked by software, the possibility of an overlap with an exotic fragment type can never be ruled out. Because the electron parity of complementary fragments in CAD is the same (even-

electron), while in ECD it is opposite (one even-electron fragment and another one odd-electron species), the problem of fragment mass overlap is more severe in CAD than in ECD [34].

IsoAsp quantification—In clinical analysis, detection of isoAsp is just a first step towards the final goal of comparing relative degrees of isomerization of a particular residue in different samples. While Asn deamidation reduces the peptide basicity and thus can alter the ionization efficiency in electrospray, isomerization of the Asp side chain has much lesser effect on ionization and for all practical purposes the ionization efficiency of isoAsp in positive ion mode is equal to that of Asp. Therefore, the relative degree of isomerization can be measured directly from the extracted ion chromatograms (EICs) without addition of an internal standard, provided the chromatographic peaks of isoAsp and Asp peptides are well separated. As an example, Figure 5A shows EICs of the peptide VLGAFSDGLAHLDNLK in two samples, one corresponding to normal brain tissue and another one – to brain tumor. The small peak at RT 557.33 is determined to be due to isomerization of Asp7 in that peptide. As an additional confirmation, ECD of a larger version of the same peptide (additional lysine is due to a missed cleavage) is shown in Figure 5C. Comparison of the chromatographic peak areas gives the isomerization degree of (3±1)% in the left EIC in Figure 5A, while in the right EIC that peak is altogether missing, which puts the isomerization degree estimate to below 0.5%. Comparison of EICs for the larger peptide gave very similar results. (data not shown)

When the chromatographic peaks of Asp and isoAsp peptides are overlapping, and ECD mass spectra contain abundant specific fragments, relative quantification can be performed by measuring the relative abundances of different ECD products. [35]

In vitro versus in vivo deamidation/isomerization—As has been shown by a large number of authors (e.g. ref. 18), many posttranslational modifications occur *in vitro* during proteomics sample handling. Deamidation is one of the most frequent *in vitro* modifications, as it is a fast, spontaneous reaction not requiring enzymatic catalysis. High pH and temperature are found to increase the rate of deamidation in overnight tryptic digestion. In this work, we did not pursue the goal of distinguishing between *in vitro* and *in vivo* formation of isoAsp. There are methodologies, e.g. the one proposed by O'Connor's group, which take advantage of isotopic labeling of peptides using $H_2\,^{18}O$ during tryptic digestion to monitor the deamidation rate *in vitro*. [36] Under certain circumstances, overnight digestion can induce up to 30% deamidation, [12] but usually less than 10% of Asn is deamidated. Since Asp isomerization is roughly around 40 times slower than Asn deamidation [32], isoAsp content exceeding 1% of that of Asp is likely to have contribution from *in vivo* isomerization processes.

Conclusion

Using the accurate masses of specific fragments of $c_n' + 57$ and $z_{l-n} - 57$, more than 460 candidates were identified in ECD mass spectra from routine proteomics experiments. Detailed examination showed, however, that many of these candidates were unreliable. To increase the specificity of isoAsp detection by ECD FTMS, a range of additional criteria were employed for candidate validation, which resulted in 219 reliable candidates. Of the criteria that were used for verification, those that can be applied to the same ECD mass spectrum (e.g. 60.0211 Da loss from the reduced species, the presence of adjacent “normal” fragments, complementary isoAsp-specific fragments, etc.) are the most suitable for incorporation into a formal search algorithm. We are currently working on such an algorithm, the challenge being the assignment of optimal weights to different criteria.

Since Asp isomerization has been reported involved in various biological processes like molecular clock, protein inactivation, protein misfolding, and degradation of protein products, implementation of automatic isoAsp identification using our findings here could potentially

help further understanding of the role in life of this non-enzymatic reaction. In both deamidation and isomerization case, care must be taken to distinguish the biological *in vivo* reaction from *in vitro* sample handling artifacts. An important moment is the quantification of the relative isomerization degree, which can be performed by the label-free approach when chromatographic peaks of aspartyl and isoaspartyl peptides are well separated.

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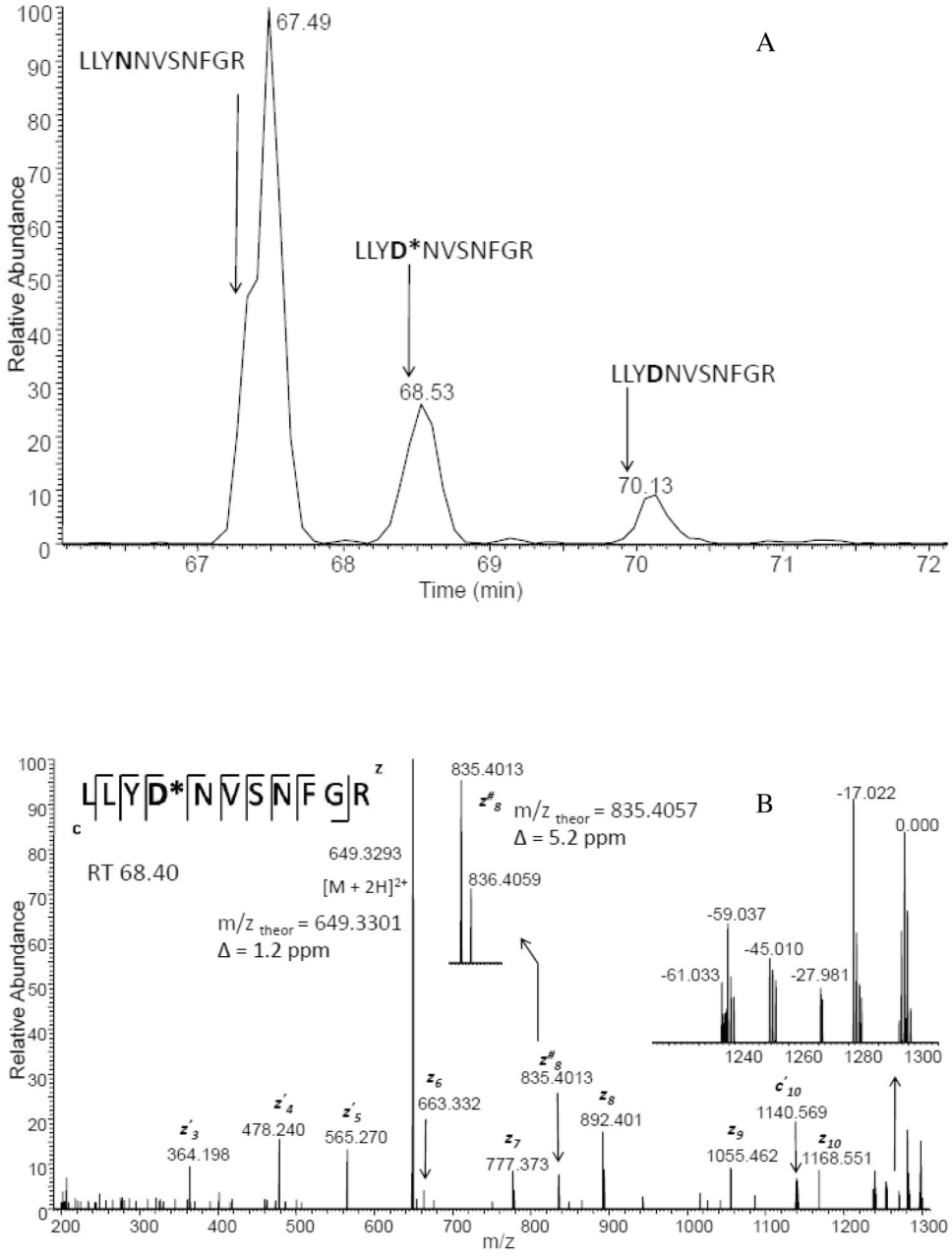


Figure 1.

A. Extracted ion chromatogram for deamidated peptide LLY(N->isoD)NVSNFGR, B. ECD MS/MS spectra for LLYisoDNVSNFGR. *D indicates the presence of isoD.

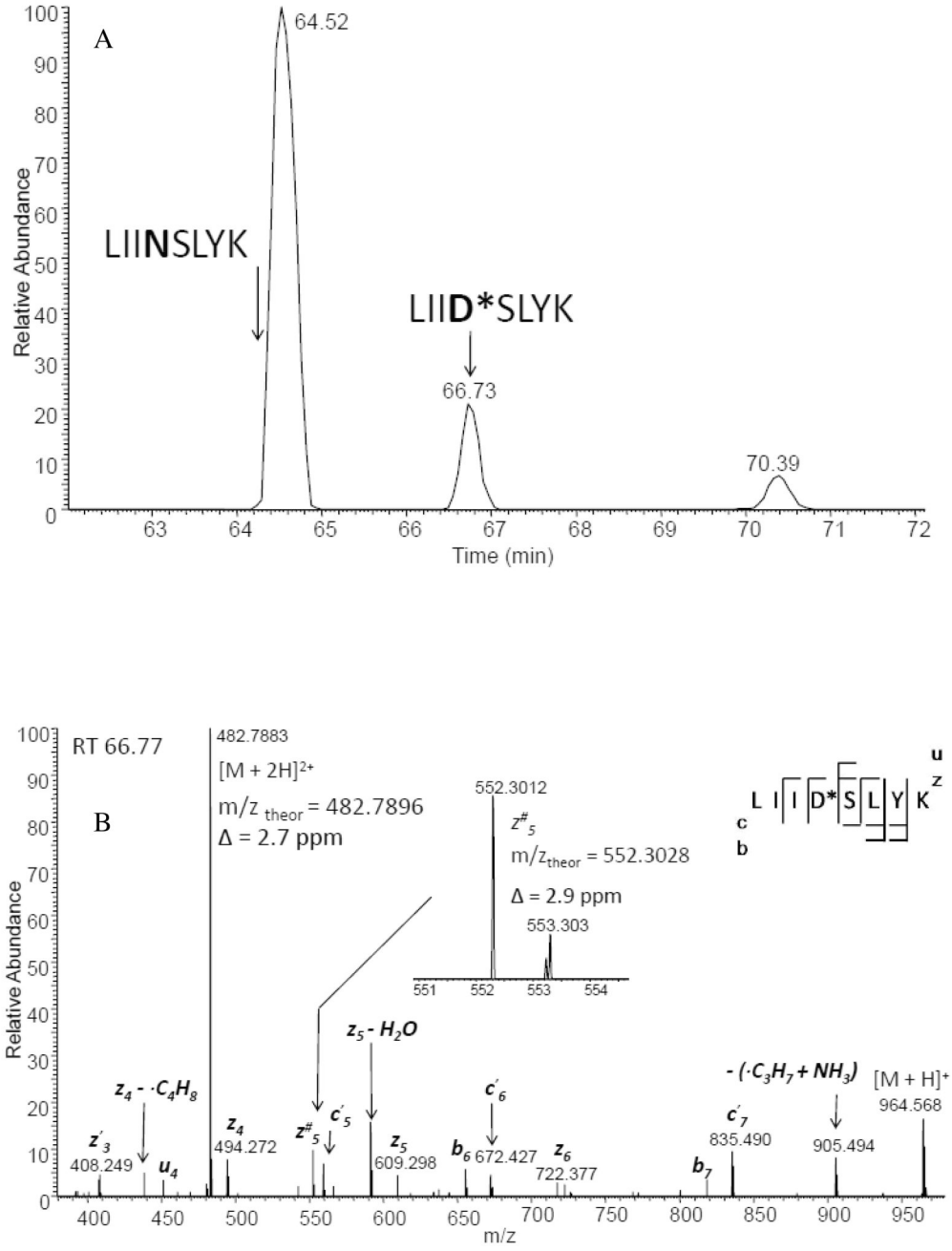


Figure 2.

A. Extracted ion chromatogram for deamidated peptide LII(N->isoD)SLYK, B. ECD MS/MS spectra for LIIisoDSLYK. *D indicates the presence of isoD.

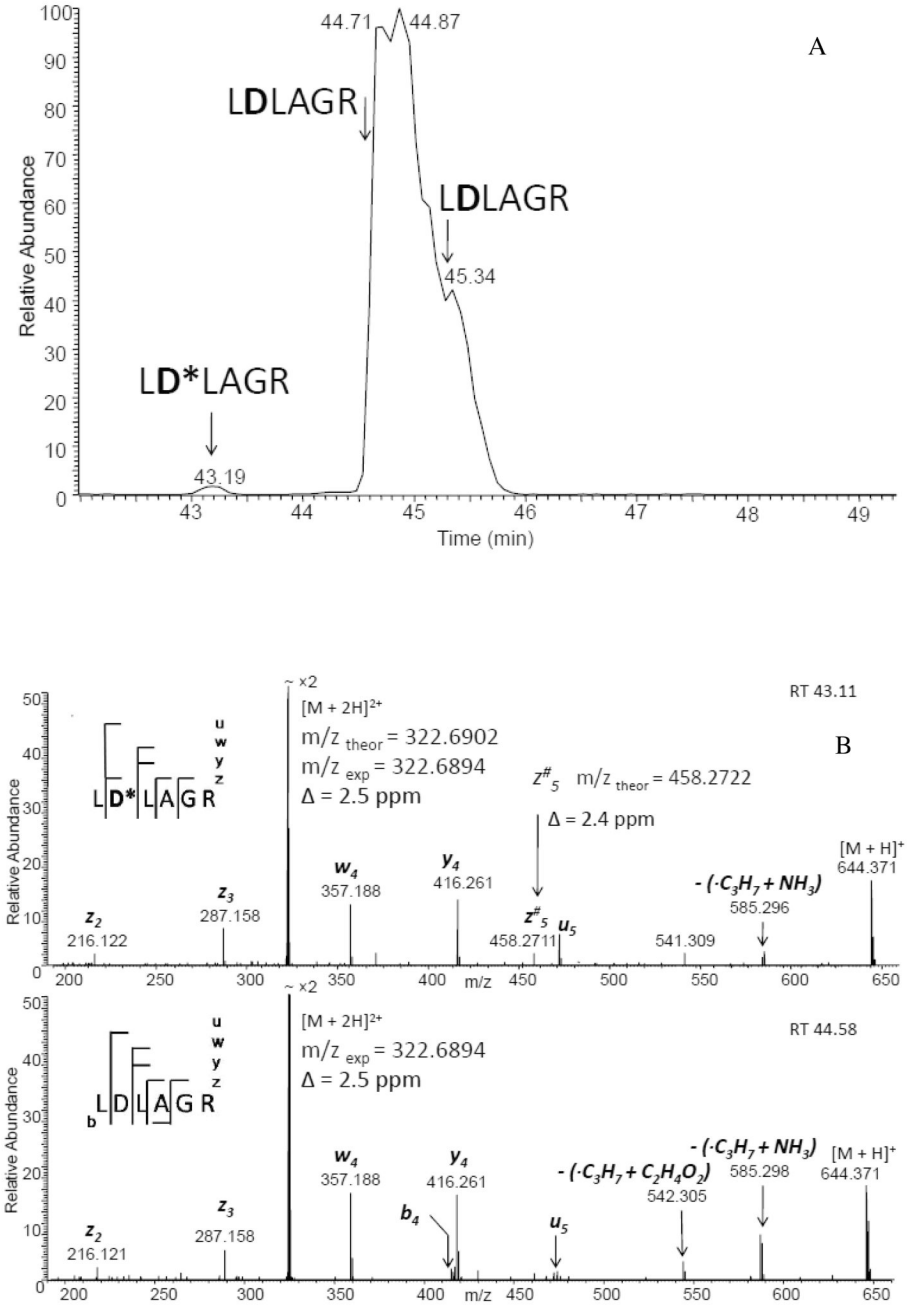


Figure 3.

A. Extracted ion chromatogram for isomerized peptide L(D->isoD)LAGR, B. ECD MS/MS spectra for LisoDLAGR(upper), and spectra for LDLAGR(lower). *D indicates the presence of isoD.

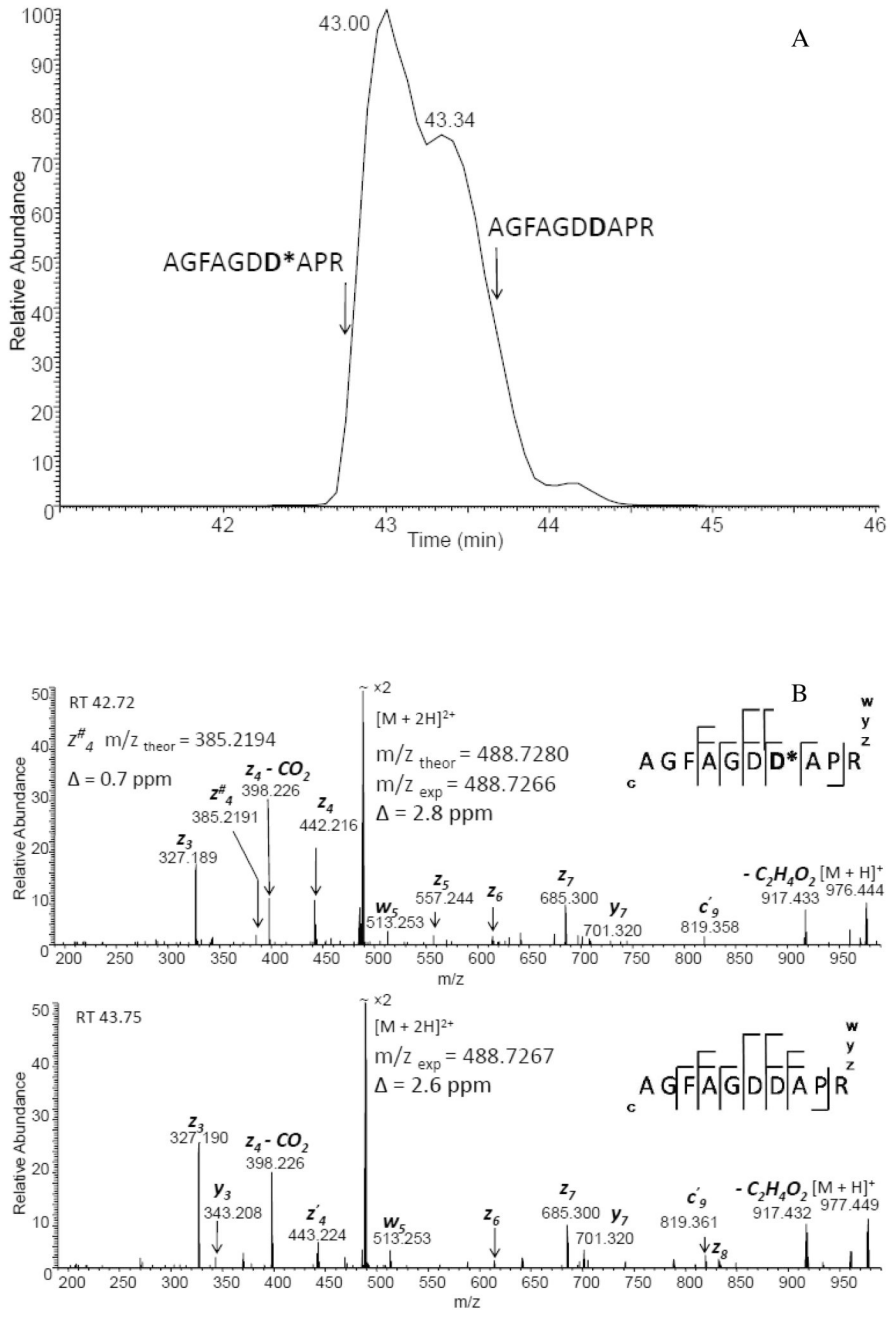
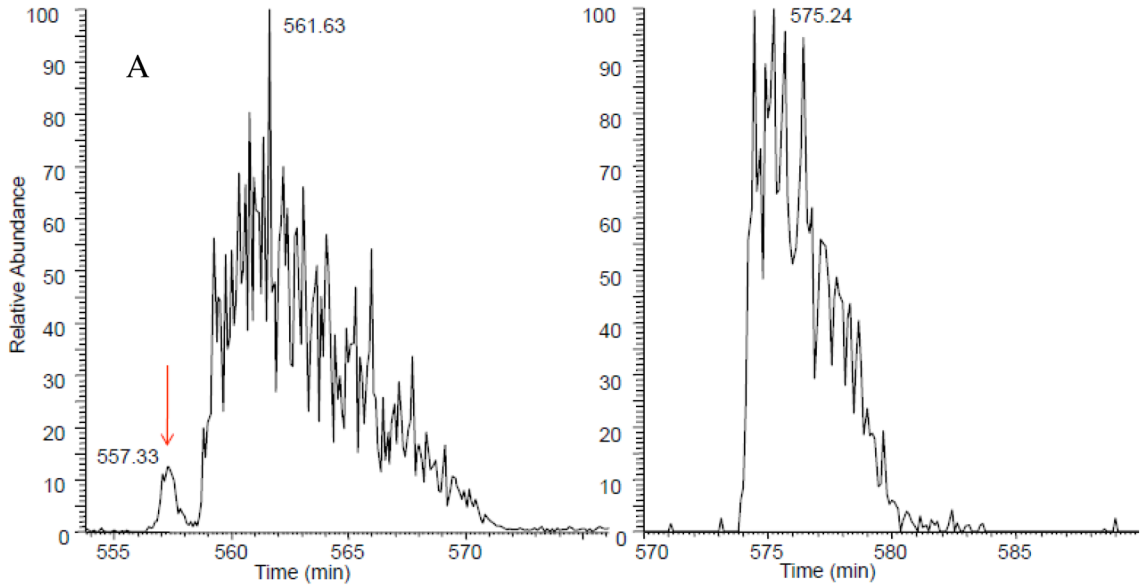
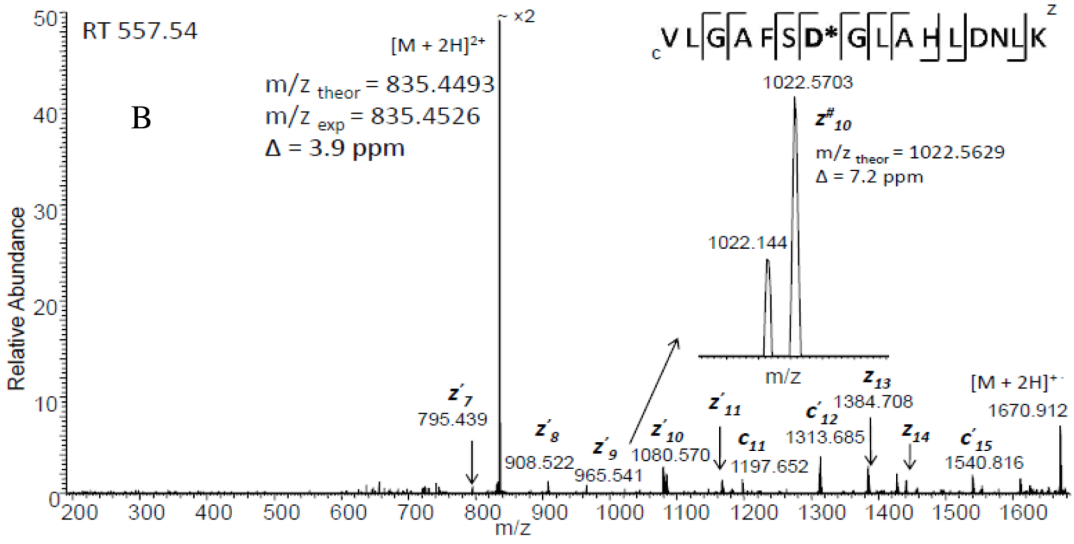


Figure 4.

A. Extracted ion chromatogram for isomerized peptide AGFAGD(D->isoD)APR, B. ECD MS/MS spectra for AGFAGDisoDAPR (upper), and spectra for AGFAGDDAPR (lower). *D indicates the presence of isoD.





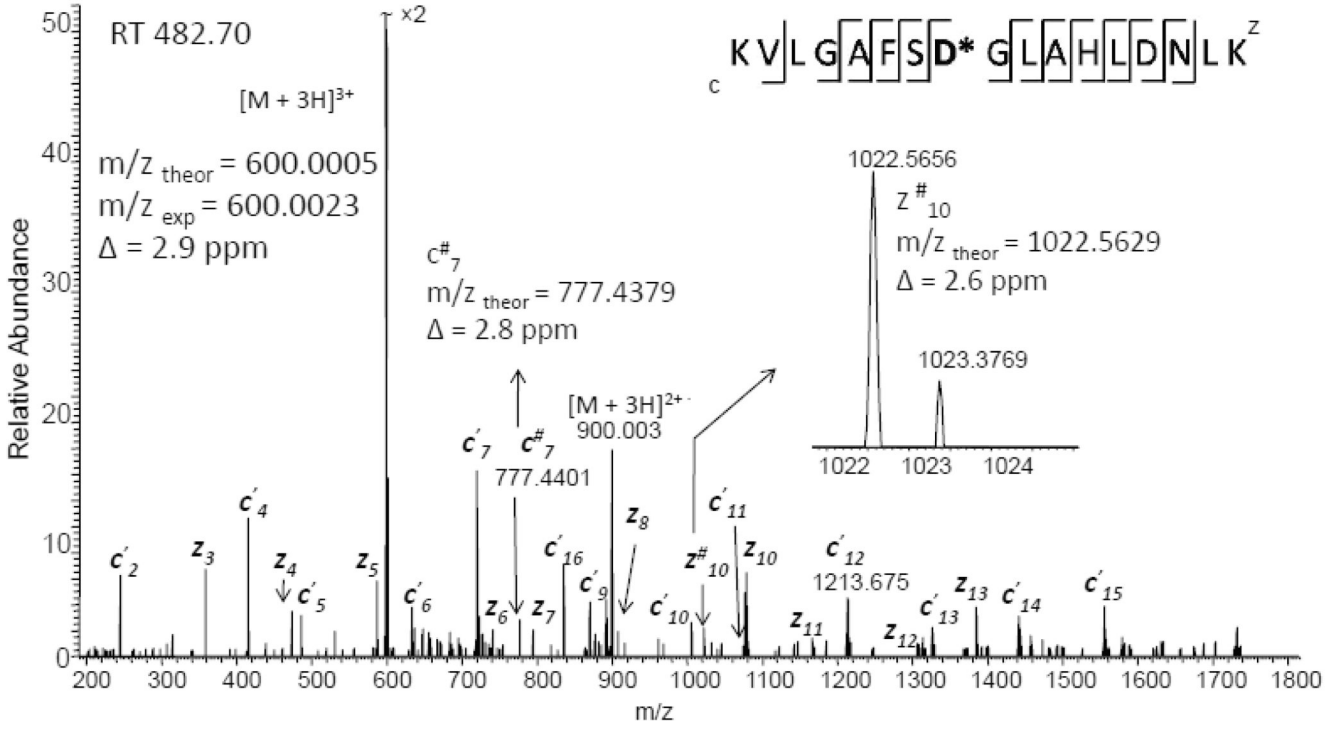
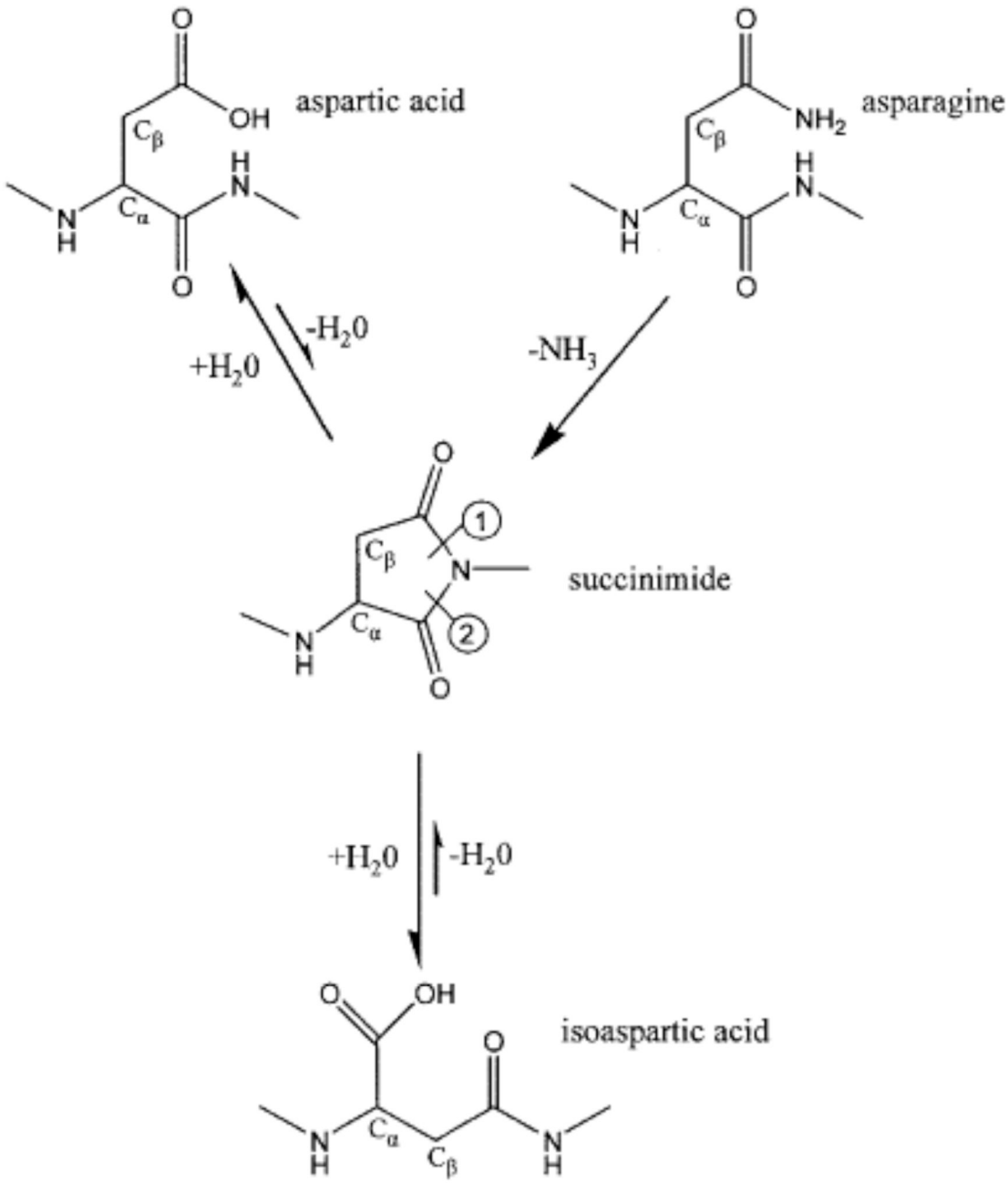


Figure 5.

A. Extracted ion chromatogram for isomerized peptide VLGAFS(D->isoD)GLAHLDNLK, left: normal brain sample, right: brain tumor sample, B. ECD MS/MS spectra for the peptide VLGAFS(D->isoD)GLAHLDNLK, C. ECD MS/MS spectra for the peptide KVLGAFS(D->isoD)GLAHLDNLK. *D indicates the presence of isoD.



Scheme 1.
Pathways of Asn deamidation and Asp isomerization through a succinimide intermediate. [13]

Table 1

Deamidated peptides, IPI number of the proteins that they are assigned to by Mascot search engine, protein names, specific fragments that have been found in the spectra, mass accuracy of the specific fragments and the presence of the adjacent fragments.

Peptide	IPI number	Protein	Specific fragment	Mass accuracy	Presence of adjacent fragments
LLY(N->D)NVSNFGR	IPI00024067	Clathrin heavy chain 1	$z^{\#}_{8}$	5.2 ppm	z_7 z_8 z_9
LII(N->D)SLYK	IPI00027230	Endoplasmin precursor	$z^{\#}_{5}$	2.9 ppm	z_4 z_5 z_6
VEIIA(N->D)DQGNR	IPI00003865	Isoform 1 of Heat shock cognate 71 kDa protein	$z^{\#}_{6}$	1.7 ppm	z_5 z_6 z_7
VGV(N->D)GFGR	IPI00219018	Glyceraldehyde-3-phosphate dehydrogenase	$z^{\#}_{5}$	1.8 ppm	z_4 z_5 z_6
VEIIANDQG(N->D)R	IPI00003362	HSPA5 protein	$z^{\#}_{2}$	5 ppm	z_2 z_3

Table 2

Isomerized peptides, IPI number of the proteins that they are assigned to by Mascot search engine, protein names, specific fragments that have been found in the spectra, mass accuracy of the specific fragments and the presence of the adjacent fragments.

Peptide	IPI number	Protein	Specific fragment	Mass accuracy	Presence of adjacent fragments
VLGAFSD*GLAHLDNLK	IPI00654755	Hemoglobin subunit beta	$z^{\#}_{10}$	7.2 ppm	z'_9 z'_{10} z'_{11}
KVLGAFSD*GLAHLDNLK	IPI00654755	Hemoglobin subunit beta	$c^{\#}_7$ $z^{\#}_{10}$	2.8 ppm 2.6 ppm	c'_6 c'_7 z_{10} z_{11}
VMDVHD*GK	IPI00384444	Keratin, type I cytoskeletal 14	$c^{\#}_5$	5.1 ppm	c'_5 c'_6
IYVDD*GLISLQVK	IPI00479186	Pyruvate kinase 3 isoform 1	$z^{\#}_9$	4.5 ppm	z_9
LD*LAGR	IPI 00021439/ IPI 00555900	Actin, cytoplasmic 1 Actin, beta-like 3	$z^{\#}_5$	2.4 ppm	u_5
AGFAGDD*APR	IPI00021439	Actin, cytoplasmic 1	$z^{\#}_4$	0.7 ppm	z_3 z_4 z_5