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Application Note 00933

The Analysis of Intact and Digested Ubiquitins on the 500-MS Ion Trap Mass Spectrometer

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Introduction

Ubiquitin is a low molecular weight heat shock protein that plays a vital role in physiological and pathological protein turnover. Recently, oxidative stress has been proposed as a pathological mechanism in Alzheimer's disease[1]. During oxidative stress in Alzheimer's disease, peroxidation products of ubiquitin are seen[2]. In vivo, the free radical super oxide reacts with nitric oxide to form peroxynitrite, a powerful oxidant. Peroxynitrite in turn reacts with ubiquitin to form the peroxidation products -- namely nitrated, oxidized and peroxidized ubiquitins[3]. These products vary depending upon the physiological pH at which the peroxidation occurs. Lack of tyrosine nitration was reported at physiological pH[4]. A direct link between ubiquitin oxidation and pathogenesis of sporadic Alzheimer's disease has been shown[5].

In the present study, peroxynitrite treated ubiquitin products were characterized on the 500-MS in two steps. Initially, intact proteins present in the peroxynitrite treated ubiquitin mixture were analyzed in full-scan mode. The multiply charged spectra obtained by the full scan MS analysis were deconvoluted to their main masses for their molecular weight confirmation. Varian's Mass ID™ software was used for the deconvolution.

In the next step, a tryptic digest of the above mixture was analyzed on the 500-MS using TurboDDS™. Protein identification was done by exporting the MS/MS spectra to the MASCOT server for a search against public databases.

Instrumentation

- Varian 500-MS LC Ion Trap equipped with an ESI source
- Varian 212-LC Binary Solvent Delivery Modules
- HTS PAL autosampler

Materials and Reagents

Ubiquitin from bovine erythrocytes (Sigma catalog no. U2153) with accessions IAAR A or IAAR B was obtained from Sigma-Aldrich, St.Louis, MO. In solution trypsin digestion kit was obtained from Pierce Biochemicals. HPLC grade water, acetonitrile, formic acid, TFA and tris buffer were purchased from Fisher Chemicals and Peroxynitrite (170-220 mM) was purchased from VWR Scientific.

Sample Preparation

Peroxynitrite treatment of ubiquitin: 5 µg/µl of ubiquitin in 50 mM tris buffer was used for peroxynitrite treatment. To 0.5 ml of 5 µg/µl solution of ubiquitin, 8 µl of 170-200 mM of peroxynitrite solution was added in aliquots of 2 µl each. This solution was vortexed at high speed and incubated at room temperature (27 ^{o}C) for 15 minutes.

Trypsin digestion of peroxynitrite treated ubiquitin was done using the digestion protocol given by the manufacturer. Protein aliquots of 10.5 µl were used for the digestion. Digestion was stopped by adding 3 µl of 100% TFA and further chilling the aliquots at 4 ^{o}C.

Conditions

LC/MS Conditions for the Analysis of Intact Proteins
(Both native- and peroxynitrite-treated ubiquitins)

Column	Pursuit™ C18 5 µm, 100 x 1.0 mm ID RP column (Varian Part No. A3000100X010)
Buffer A	1% formic acid in water
Buffer B	1% formic acid in acetonitrile (CH_3CN)
Injection Solvent	Buffer A
Injection Volume	10 µl
LC Program	Analysis of Intact Proteins

Time (min:sec)	%A	%B	Flow (µl/min)
0:00	80.0	20.0	60.0
7:00	0.0	100.0	60.0
7:01	80.0	20.0	60.0
15:00	80.0	20.0	60.0

Full Scan MS Conditions

Scan Range	*m/z*=350 to *m/z*=2000
RF Loading %	100%
Scan Mode	Enhanced (5000 amu/sec)
Scans averaged	10 µscans

Common API Conditions
(Full-scan and data-dependent modes)

Ionization Mode	ESI Positive
API Drying Gas	30 psi at 250 ^{o}C
API Nebulizing Gas	45 psi
Needle	5000 V
Capillary	80 V
Shield	600 V
Detector	1400 V

LC/MS Conditions for the Analysis of Ubiquitin Tryptic Digest

Column	Pursuit™ XRs C18 5 µm, 150 x 2 mm ID RP column (Varian Part No. A6000150X010)
Buffer A	1% formic acid in water
Buffer B	1% formic acid in acetonitrile (CH_3CN)
Injection Solvent	Buffer A
Injection Volume	10 µl
LC Program	Analysis of Tryptic Digests

Time (min:sec)	%A	%B	Flow (µl/min)
00:00	95.0	5.0	200.0
50:00	0.0	100.0	200.0
51:00	95.0	5.0	200.0
60:00	95.0	5.0	200.0

TurboDDS™ Conditions

Survey Scan	Range: *m/z*=200 to *m/z*=2000 RF loading: 100%
TurboDDS MS^n	MS^n depth n: 2 Mass range: Auto Isolation window: 3.0 *m/z*
MS2 Parameters	Breadth n: 5 µscans: 3 Mass range: *m/z*=50 –*m/z*=2000 Threshold: 500 counts
TurboDDS Trigger	Threshold: 500 counts Isotoptic range: 3 *m/z* Minimum isotopic ratio: 30% Filter width: 7 points
TurboDDS Include/Exclude	Dynamic exclusion: yes Exclude after: 2 scans Restore after: never restore

Data Processing

Intact protein molecular weights were determined by deconvolution (Varian's Mass ID™ software). The protein digest data was searched using MASCOT against the MSDB database.

Results & Discussion

Initially, peroxynitrite treated ubiquitin was analyzed by full-scan LC/MS on the 500-MS (Figure 1). Charge states from +5 to +14 were observed in a mass range of 500-2000 *m/z*. The averaged full scan MS spectrum was deconvoluted using the Mass ID software. The original full scan MS spectrum and deconvoluted spectra are shown in Figures 2 and 3, respectively. As seen from the deconvoluted spectra, two other major proteins were found in addition to the unmodified ubiquitin. These proteins have a mass difference of +15.5 and + 44.8 from the unmodified ubiquitin corresponding to its oxidized and nitrated forms, respectively. Excellent deconvolution was obtained for the native and modified ubiquitins. Extremely low mass errors (< 0.02%) indicate the accuracy of deconvolution. These modified proteins co-eluted along with the native protein under the applied LC/MS conditions.

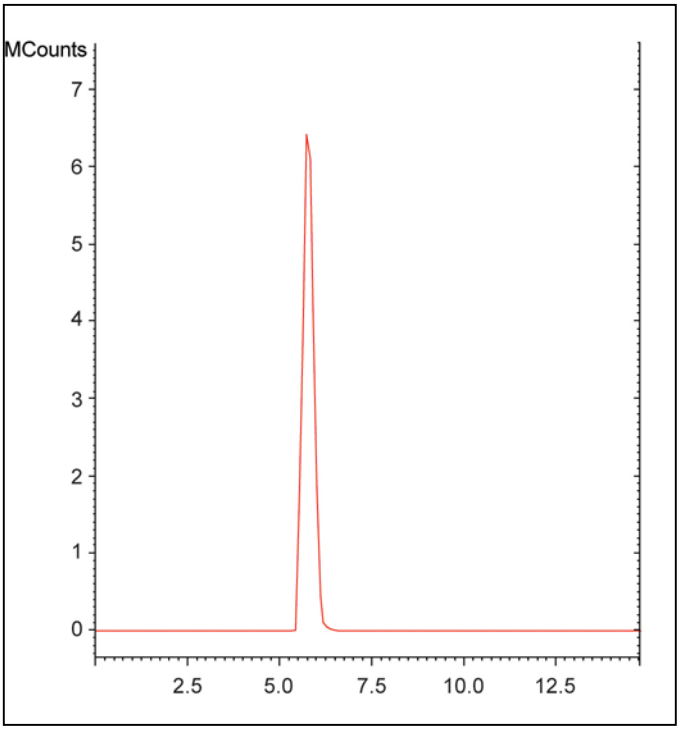


Figure 1 Full scan MS TIC chromatogram of peroxynitrite treated ubiquitin mixture (8 pmoles/µl, 10 µl injection) on the 500-MS.

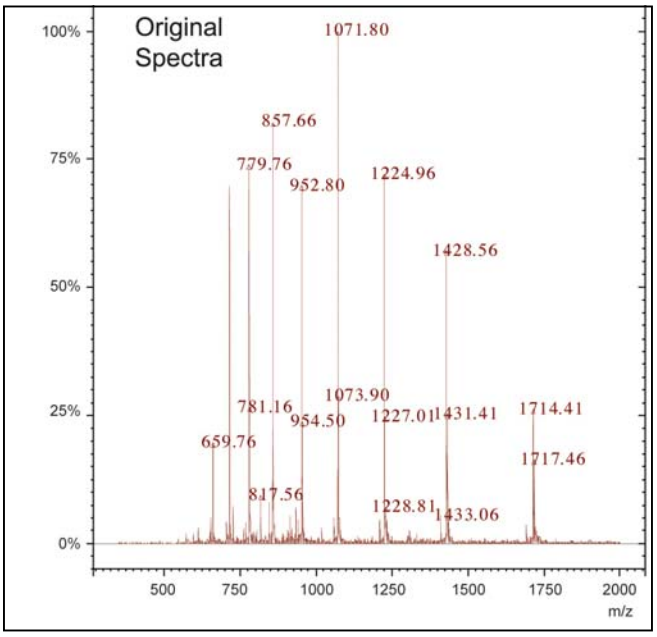


Figure 2 Original spectra of the peroxynitrite treated ubiquitin obtained by LC/MS on the 500-MS.

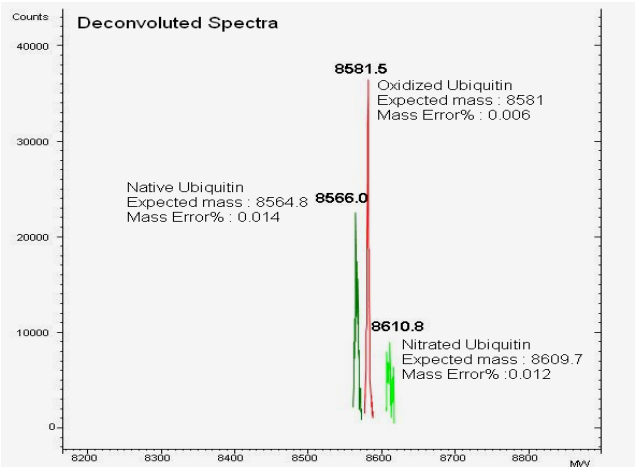


Figure 3 Deconvoluted spectra of the peroxynitrite treated ubiquitin.

In order to further identify the protein, a tryptic digest of this mixture was analyzed by employing the TurboDDS™ option of the 500-MS. The results generated were exported to the MASCOT server to search against the MSDB database. The MASCOT search identified the protein as ubiquitin with peptide sequence coverage of 92% (Figure 4). Despite the low concentration (8.4 picomoles/µl), excellent sequence coverage was obtained. As seen from the MASCOT search results (Figure 4) this sequence is identical in humans, bovine erythrocytes (IAAR A & IAAR B) and several other species. This confirms the source of ubiquitin.

Protein view

Match to: **UQHU** Score: **68**
Ubiquitin - human (tentative sequence)
Found in search of C:\Documents and Settings\mrudrabh\Desktop\Ubiquitin searches\previous mascot searches\MGF files\Ub dig SDS 3 us.MGF

Nominal mass (M_r): **8560**; Calculated pI value: **6.56**
NCBI BLAST search of UQHU against nr

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:

1D3ZA from Homo sapiens
1AARA from Bos taurus (ubiquitin from bovine erythrocytes)
1AARB from Bos taurus (ubiquitin from bovine erythrocytes)
1TBEA from Homo sapiens

Variable modifications: Oxidation (M), Nitro (W), Nitro (Y).
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **92%**

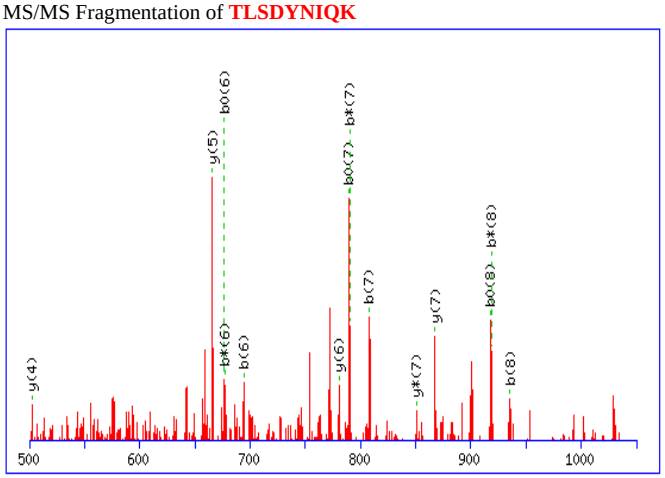
Matched peptides shown in **Bold Red**

1 **MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL**
51 **EDGRTLSDYN IQKESTLHLV LRLRGG**

Figure 4 Protein view of MASCOT search results for ubiquitin tryptic digests against MSDB database.

Peptide View

MS/MS Fragmentation of **TLSDYNIQK**



Monoisotopic mass of neutral peptide Mr(calc): 1080.55
Ions Score: 16 **Expect:** 33
Matches (Bold Red): 14/38 fragment ions using 46 most intense peaks

#	b	b*	b^0	Seq.	y	y*	y^0	#
1	102.05		84.04	T				9
2	215.14		197.13	L	980.50	963.48	962.49	8
3	302.17		284.16	S	**867.42**	**850.39**	849.41	7
4	417.20		399.19	D	**780.39**	763.36	762.38	6
5	580.26		562.25	Y	**665.36**	648.34		5
6	**694.30**	**677.28**	**676.29**	N	**502.30**	485.27		4
7	**807.39**	**790.36**	**789.38**	I	388.26	371.23		3
8	**935.45**	**918.42**	**917.44**	Q	275.17	258.14		2
9				K	147.11	130.09		1

Figure 5 Peptide view for TLSDNIQK on MASCOT server.

The peptide view of the MASCOT search for the peptide TLSDNIQK is given in Figure 5. In the peptide view, fragments that might result due to MS/MS breakdown are predicted. The fragments that matched the exported MS/MS spectra are highlighted in red.

Conclusion

Molecular weight confirmation of peroxynitrite-induced modification of ubiquitin products was very effectively done with the help of full-scan MS coupled with Varian's Mass ID™ deconvolution software.

TurboDDS provided complex MS/MS spectra of the protein without significant user interaction.

Excellent peptide sequence coverage (92%) at low concentrations (8.4 pmoles/µl) was obtained on the MASCOT server by exporting the MS/MS spectra generated on the Varian 500-MS using the powerful TurboDDS option.

References

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