SHIMADZU APPLICATION NEWS

LAAN-A-LM-E012

● LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

No.C55

Analysis of Proteins and Peptides using LC-MS

Proteins, one of the key constituents of living organisms, are created in the body from amino acids, and play an important role in the maintenance (and other functions) associated with antibodies, enzymes, hormones, receptors and body structure. Examples of the use of proteins in the industrial world include, besides the nutritional standpoint, an ever-expanding list of products from enzymes for detergents to medicines for treatment of diseases.

Previously, medicines made from proteins used purified proteins from the body. Recently, however these protein medicines are being produced by applying recombinant technology, bringing such proteins as insulin, interferon, and erythropoietin, etc. to the market. From the standpoint of quality control, expression verification of a recombinant protein is an important process, and this is currently conducted using such technologies as peptide sequencing for amino acid analysis, HPLC for simple peptide mapping, and MALDI-TOFMS for mass mapping, etc.

This Application News introduces examples of LC-MS for protein analysis, in which the LCMS-2010EV quadrupole mass spectrometer was used for the intact analysis of myoglobin from horse heart as the model sample, and for peptide mass mapping.

Fig.1 shows the positive electrospray ionization (ESI) mass spectrum of myoglobin. Infusion analysis of the purified protein (5 % aqueous acetic acid and 50 % methanol solution) was conducted using a syringe pump. Multiply charged ions with 9 to 21 charges were detected in the *m/z* 700 to 2000 range.

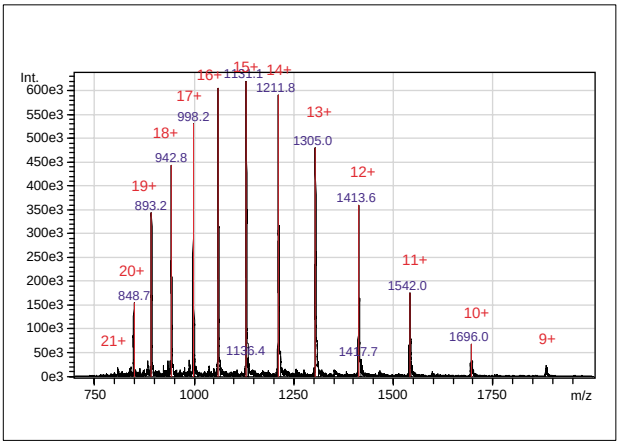

Fig.1 ESI MS Spectrum of Horse Heart Myoglobin

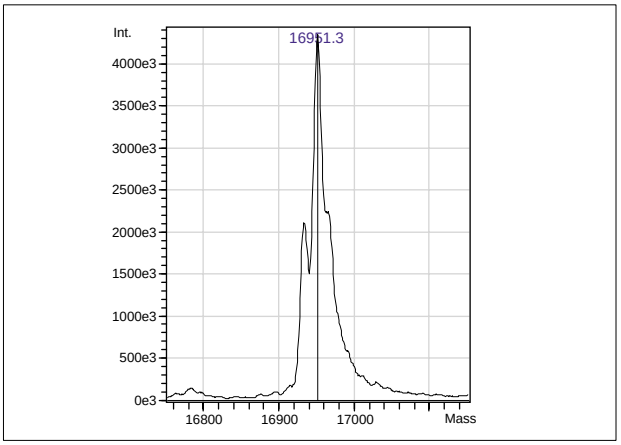

Fig.2 Deconvoluted Average Mass Spectrum of Apomyoglobin

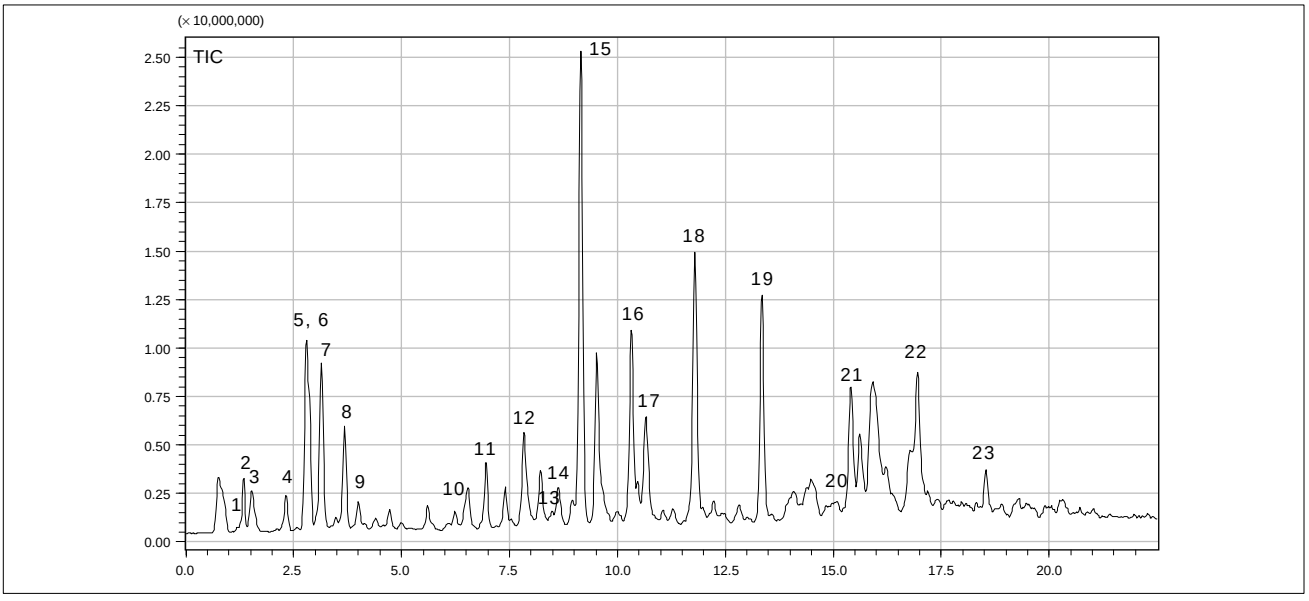

Fig.3 Total Ion Chromatogram of Tryptic Digest of Horse Heart Myoglobin

When multiply charged ion mass spectra are converted into a neutral mass spectrum (Fig.2) by algebraic deconvolution, the molecular weight of myoglobin is calculated to be 16951. An excellent result is obtained in comparison with the theoretical molecular weight of 16951.5 (average mass) calculated from the amino acid composition of apomyoglobin. In this way, when the target compound has multiple ionized sites and is detected as a multiply charged ion using ESI, it is possible to analyze proteins which have a singly charged state that exceeds the measurement range of the mass spectrometer.

Enzymatic digestion was conducted using TPCK trypsin after denaturation of the myoglobin, followed by electrospray – LC-MS analysis using an ODS column. Fig.3 shows the total ion chromatogram (TIC) of the tryptic digest. Approximately 40 peaks were detected. Fig.4 (a) – (f) shows the mass spectra of the typical digest peaks.
In peak 4, ions *m/z* 316 and 631, which belong to the doubly charged protonated molecule $[M+2H]^{2+}$ and singly charged protonated molecule $[M+H]^{+}$, respectively, were detected (Fig.4a). As a result, the molecular weight of this peptide is calculated to be 630. Similarly, the molecular weight calculated from the mass spectrum of peak 5 is 2109, of peak 8, 1853, of peak 15, 1936, of peak 19, 1378, and of peak 21, 3384.

Table 1 lists the detected ions, the theoretical *m/z* values of the corresponding tryptic digest peptide ions, and the peptide molecular weight with respect to each peptide peak. Trypsin is a protease that cuts the C terminal side of lysine and arginine in the protein, and this enzymatic digestion allows prediction of the peptides generated from the amino acid sequence of the protein (right column in Table 1). By comparing the peptide of peak 4 with the primary structure of myoglobin, it is found to correspond to T140 – 145 (N terminal number 140 to 145). In this analysis, the peptides with molecular weights from 600 to 3500 were confirmed, and it was possible to correlate the respective amino acid sequences of the tryptic digest peptides.

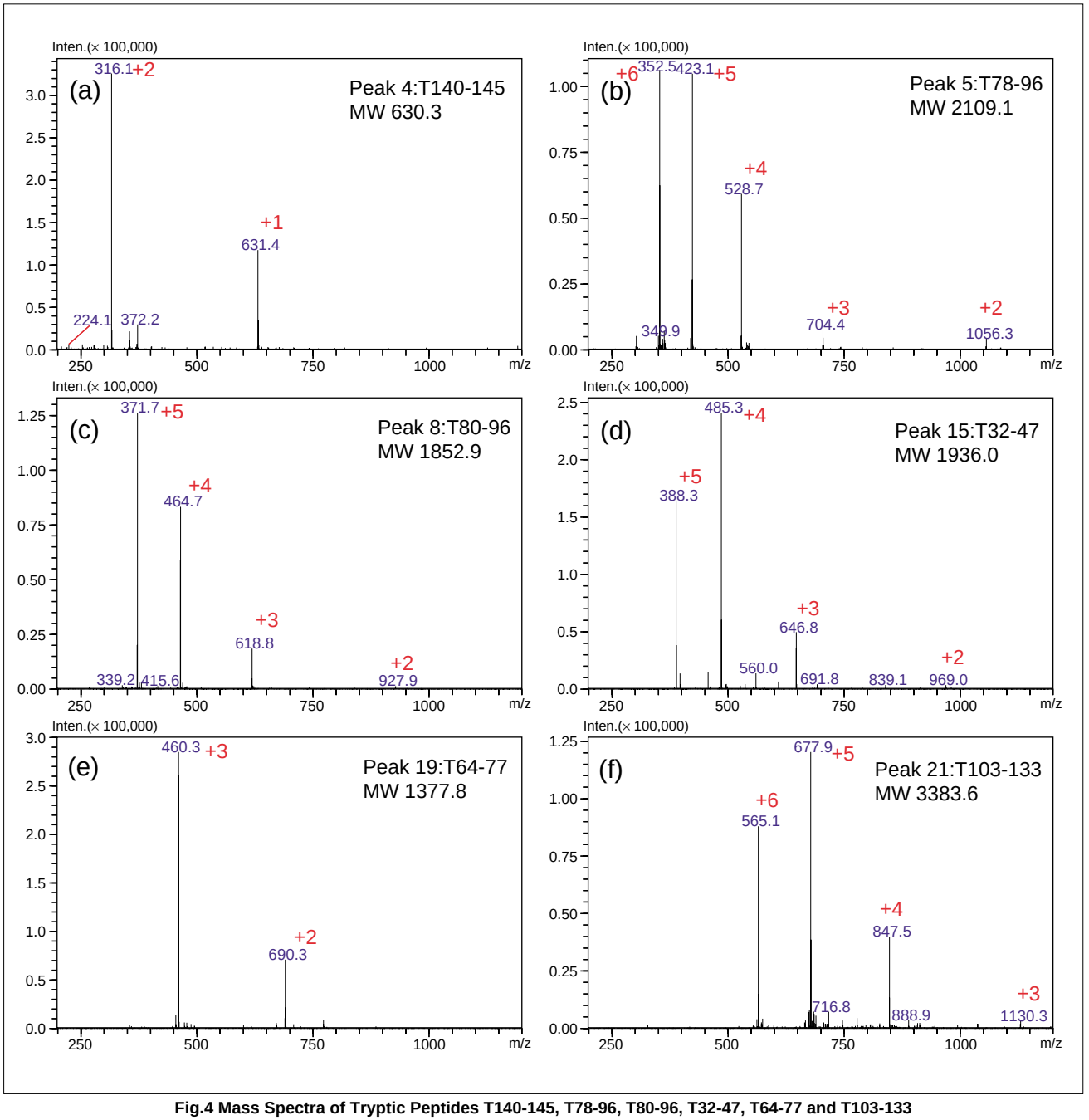

Fig.4 Mass Spectra of Tryptic Peptides T140-145, T78-96, T80-96, T32-47, T64-77 and T103-133

Table 1 Detected Peptides of Tryptic Myoglobin

No	R.T. (min)	Detected ions (*m/z*)	Theoretical *m/z* value (charge state)	Molecular weight	Estimated peptides (sequence)
1	1.2	363 724	362.7 (+2) 724.3 (+1)	723.3	T51-56 : TEAEMK
2	1.3	368 552	368.1 (+3) 551.8 (+2)	1101.5	T48-50+T51-56 : HLKTEAEMK
3	1.5	264 396 790	264.1 (+3) 395.7 (+2) 790.4 (+1)	789.4	T57-62+T63 : ASEDLKK
4	2.3	316 631	316.2 (+2) 631.3 (+1)	630.3	T140-145 : NDIAAK
5	2.7	352 423 529 704 1056	352.5 (+6) 422.8 (+5) 528.3 (+4) 704.0 (+3) 1055.6 (+2)	2109.1	T78+T79+T80-96 : KKGHHEAELKPLAQSHATK
6	2.8	246 368 735	245.8 (+3) 368.2 (+2) 735.5 (+1)	734.5	T97-98+T99-102 : HKIPIK
7	3.1	397 496 661 992	397.2 (+5) 496.3 (+4) 661.3 (+3) 991.5 (+2)	1981.0	T79+T80-96 : KGHHEAELKPLAQSHATK
8	3.6	372 465 619 927	371.6 (+5) 464.2 (+4) 618.7 (+3) 927.5 (+2)	1852.9	T80-96 : GHHEAELKPLAQSHATK
9	3.9	229 343 684	228.8 (+3) 342.7 (+2) 684.4 (+1)	683.4	T43-45+T46-47 : FDKFK
10	6.2	501 752	501.5 (+3) 751.8 (+2)	1501.7	T119-133 : HPGDFGADAQGAMTK
11	6.9	424 636	424.5 (+3) 636.3 (+2)	1270.6	T32-42 : LFTGHPETLEK
12	7.8	471 941	471.2 (+2) 941.4 (+1)	940.4	T146-147+T148-153 : YKELGFQG
13	8.3	650	650.3 (+1)	649.3	T148-153 : ELGFQG
14	8.6	536 804	536.3 (+3) 803.9 (+2)	1605.8	T17-31 : VEADIAGHGQEVLIR
15	9.2	388 485 647 969	388.2 (+5) 485.0 (+4) 646.3 (+3) 969.0 (+2)	1936.0	T32-42+T43-45+T46-47 : LFTGHPETLEKFDKFK
16	10.3	454 681	454.2 (+3) 680.9 (+2)	1359.7	T134-139+T140-145 : ALELFRNDIAAK
17	10.6	375 748	374.7 (+2) 748.4 (+1)	747.4	T134-139 : ALELFR
18	11.8	377 503 754	377.4 (+4) 502.9 (+3) 753.9 (+2)	1505.91	T63+T64-77 : KHGTVVLTALGGILK
19	13.4	460 690	460.3 (+3) 689.9 (+2)	377.8	T64-77 : HGTVVLTALGGILK
20	15.3	606 909	605.9 (+3) 908.4 (+2)	1814.9	T1-16 : GLSDGEWQQVLNVWGK
21	15.7	565 678 847 1129	564.9 (+6) 677.7 (+5) 846.9 (+4) 1128.9 (+3)	3383.6	T103-118+T119-133 : YLEFISDAIIHVLHSKHPGDFGADAQGAMTK
22	16.9	472 629 943	472.0 (+4) 629.0 (+3) 943.0 (+2)	1884.0	T103-118 : YLEFISDAIIHVLHSK
23	18.5	852 1135	851.7 (+4) 1135.3 (+3)	3402.7	T1-16+T17-31 : GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR

The peptides detected here were confirmed, with the exception of free lysine, to cover all of the amino acid regions of myoglobin (Fig.5). From the above results, it is clear that ESI – LC-MS proves useful for confirmation of recombinant protein expression, confirmation of structural changes occurring within structurally known peptides, and as a means of obtaining molecular weight information for supplementing peptide sequence data.
Leucine and isoleucine, which have exactly the same molecular weight, cannot be distinguished using mass measurement of the amino acid alone. (These can be distinguished using higher order MS fragmentation functions, however.) Moreover, the resolution of the single quadrupole MS makes it difficult to distinguish between glutamine and lysine, which have a mass difference of 0.04 Da. Use of an amino acid composition analysis method in conjunction with MS analysis is effective for determining the compositions of the peptides included in these amino acids. Furthermore, an MS/MS instrument such as the LCMS-IT-TOF hybrid mass spectrometer is effective for *de novo* sequence analysis of structurally unknown peptides.

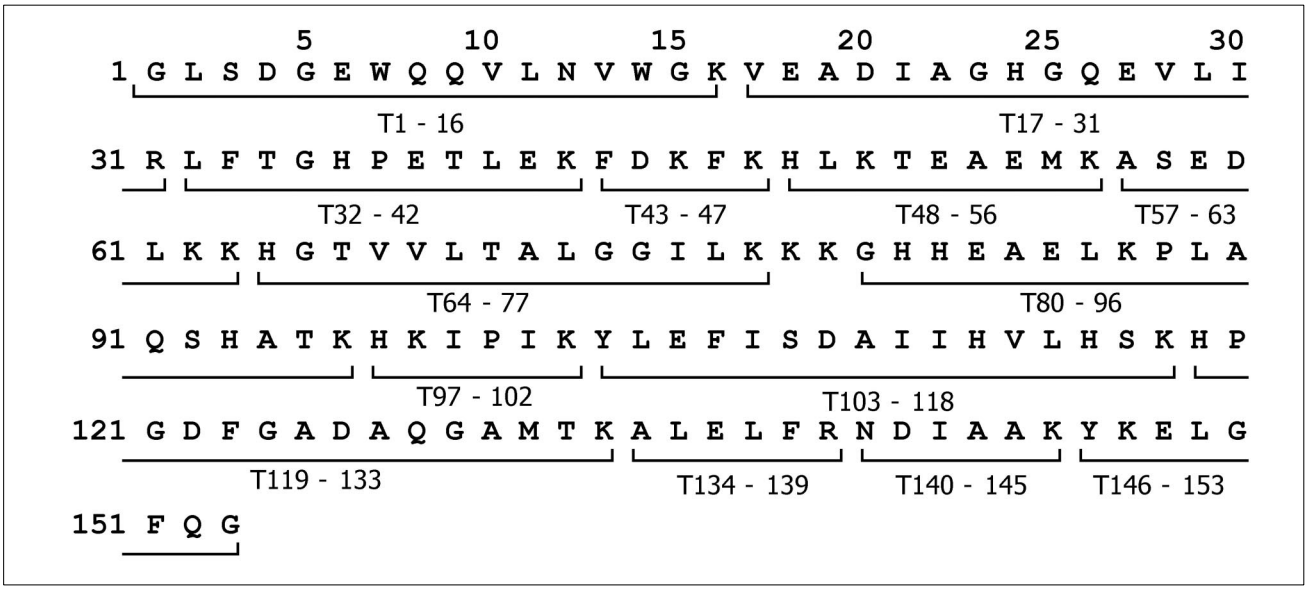

Fig.5 Amino Acid Sequence of Horse Heart Myoglobin

Table 2 LC-MS Analytical Conditions

Column	: Imtakt Cadenza CD-C18 (1.0 mmI.D. × 75 mmL.)		
Mobile phase A	: 0.1 % formic acid - water		
Mobile phase B	: 0.1 % formic acid - 70 % acetonitrile		
Time program	: 5 %B (0 min)→80 %B (30 min)		
Flow rate	: 0.05 mL/min		
Injection volume	: 5 µL	Column temperature	: 40 ˚C
Probe voltage	: +4.5 kV (ESI-Positive mode)		
CDL temperature	: 250 ˚C	Block heater temperature	: 200 ˚C
Nebulizing gas flow	: 1.0 L/min		
Drying gas pressure	: 0.02 MPa		
CDL voltage	: + 10 V		
Q-array DC voltage	: Scan-mode	Q-array RF voltage	: Scan-mode
Scan range	: *m/z* 200-1200 (1.0 sec/scan)		

NOTES:
✻This Application News has been produced and edited using information that was available when the data was acquired for each article. This Application News is subject to revision without prior notice.

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