EXTRACTION AND MS-MS STUDY OF MYOGLOBINS FROM VARIOUS SPECIES

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SUMMARY

Studies on the heat stability of myoglobin and haemoglobin from sheep in model systems showed that myoglobin globin was more stable than haemoglobin (Ponce *et al.,* 1994). In heated meat and meat products, only globins from myoglobin were detected in the extract and no sign of haemoglobin could be found, suggesting it had been degraded. ESMS has potential as a rapid and sensitive method not just to measure the accurate molecular mass but also to detect myoglobin in simple aqueous model systems, and in rather more complex systems like cooked meat and meat products (Ponce *et al.,* 1995). However when mixtures of beef-pork and beef-lamb were analysed by ESMS just the presence of one species was observed. The differences between Beef, Horse and Pork myoglobins are too close in mass to be differentiate by mass alone. ESMS production of molecular ions followed by Collision-Induced-Dissociation (CID) fragmentation may be used to obtain structural information from intact proteins more rapidly and sensitively than with other conventional techniques. The differences in the amino acid sequence of myoglobin within species, may originate different fragmentation patterns that, could be used as a tool for species differentiation by performing an MS/MS analysis.

Species identification could be achieved by two steps; first as the molecular mass of sheep myoglobin is 16923.4 Da; it may be differentiate from beef, horse and pork by ESMS, because it is ≈23Da lower in mass than the other three species. Also pork and beef (16953.5-16946.4 = 7.1 Da) may be differentiated by the same method. The difference in molecular weight for horse and beef is 16951.5-16946.4 = 5.1 Da, while for pork an horse 16953.5-16951.5 = 2 Da. In theory with a resolution of 0.01% and a molecular mass of 16950 Da, differences of 1.695 Da can be resolved. However the molecular mass are too close in mass to be differentiate by mass alone. Thus beef can be differentiated from pork and horse by MSMS analysis by detection of the y^{5+}_{34} and y''^{4+}_{34} fragments.

MATERIALS AND METHODS

Myoglobins from various species were extracted following Yamazaki (1964) and Satterlee (1972) by precipitation with ammonium sulphate from 65% up to 100% of saturation, the resulting precipitated was collected, redissolved and purified on DEAE-cellulose column anion exchange chromatography.

Sample solutions were assayed on a VG Quatro II mass spectrometer fitted with a electrospray interface (VG Biotech, Altrincham, UK). The solvent stream consisted of 50% aqueous methanol and 0.2% of formic acid solution was pumped via Rheodyne valve into the source at 5 μl min^{-1}.The inlet capillary was maintained at 4 kV with the counter electrode at 1 kV and the source temperature at 70°C. The ESMS raw spectra were obtained in the first mass spectrometer by taking 10-15 scans. The molecular mass was determined by transformation of the ESMS raw data on a true molecular mass scale containing a single peak of each compound present in the sample. The mass scale was calibrated using horse heart myoglobin (Sigma: 16951.51 Da, Zaia *et al.*, 1992).

The MSMS analysis was achieved by selecting the precursors ions $[M+17H]^{17+}$ ($m/z \approx 998$) and $[M+16H]^{16+}$ ($m/z \approx 1060$) in the first mass spectrometer, then fragmentation was performed by transferring the selected ions into the hexapole collision cell, using helium as the collision gas. The product ions were then transported into the second mass spectrometer which separates them according to their mass to charge ratio (*m/z).*

RESULTS AND DISCUSSIONS

Myoglobin contains a single polypeptide chain of 153 amino acid residues and a single iron porphyrin or heme group noncovalently bound to the peptide chain, during ionisation myoglobin is dissociated into the globin chain and the heme and detected as different entities. The measured molecular weight of the myoglobin globin chain for all samples (Table 1) agree within the experimental error with the molecular mass calculated from the amino acid sequences (EMBL library). ESMS produces intact multiply protonated protein ions, the ESMS spectra shows a bell-shaped series of multiply charged protein ions peaks. Each peak differs from the adjacent in one charge and any two consecutive ions can be used for the accurate determination of charge and mass. ESMS spectras showed myoglobin as the principal component of this fraction, high molecular components and sodium adduct series (M+Na, M+2Na, M+3Na, etc.) were present; adducts probably derived from the buffers used during the DEAE cellulose chromatography.

On the MSMS analysis two consecutive mass analysers are used, the first generates and selects the precursor ion (or parent ion) from the peptide, which then passes through a collision cell where fragmentation takes place. The product ions are then analysed in a second mass spectrometer and the MSMS spectra is recorded. Because of this instrument arrangement, the method is called MSMS.

CID induces fragmentation (Biemann *et al.*, 1987) in which the selected parent ion collides with a neutral atom such as helium, increasing the internal energy. Ions that undergone this collisional excitation process may subsequently fragment. Highly charged molecules are more susceptible to dissociation because the collision energy is proportional to the number of charges at a given *m/z*, also dissociation is enhanced by the presence of electrostatic repulsive forces (Tang *et al.,* 1993).

Any ion in the series could be used as a parent ion for the MSMS analysis. The precursors ions selected for the MSMS analysis were $[M+17H]^{17+}$ ($m/z \approx 998$) and $[M+16H]^{16+}$ ($m/z \approx 1060$) from the MS data obtained in the first mass spectrometer. For each ion in the ion product mass spectra all the *m/z* values and all the possible charge states are compared to all possible b and y fragments following

the Roepstorff *et al.* (1984) nomenclature from the known amino acid sequence. The resultant MSMS spectra from the four myoglobin globin species were compared and all were very similar and complex, dominated by series of y"$_n$ fragments. The fragments produced by cleaving 119/120 (His/Pro) and 99/100 (Ile/Pro) residues are relative intense (y"$_{34}$ and y"$_{54}$). Loo *et al.* (1990, 1993) suggested that dissociation is commonly detected on the amide bond of the proline residue and it is more significant as the molecular mass increases. Almost 80% of the myoglobin chain is α-helical, the formation bends in the polypeptide chain direction and angle is determined by the location of proline, theonine or serine residues; proline prevents rotation of the N-C$^{\alpha}$ bond of the backbone having a very significant effect on the conformation of the polypeptide and therefore on the MSMS fragmentation process.

The most evident differences between the spectra were observed in the y"$_{34}$ fragment derived from the $[M+17H]^{17+}$ precursor ion. The predicted *m/z* values for this fragment are summarised in Table 2. The predicted *m/z* value for y"$^{5+}_{34}$ fragment from sheep (728 *m/z* units) and pork (728 *m/z* units) globins are identical. However this fragment is not observed in the sheep myoglobin spectra, because it has less number of charges *ie* less number of basic amino acids. The values for horse and pork myoglobin globins are very close (Δ=0.8 *m/z* units). On the other hand the fragment derived from pork myoglobin is less intense, compared with those from horse and beef samples.

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TABLE 1. Molecular weight of Myoglobin from different species

Specie	Molecular mass (Da)	
	Calculated	ESMS data ± S.D.
Sheep	16923.4	16923.4 ± 0.4
Beef	16946.4	16946.6 ± 0.5
Horse	16951.5	16951.5*
Pork	16953.5	16953.5 ± 0.2

* Mass scale was calibrated using horse myoglobin.

TABLE 2. MSMS predicted *m/z* values and number of charged sites for y^{n+}_{34} fragment.

Specie	Charged sites	Fragment Y^{5+}_{34}		Fragment Y^{4+}_{34}	
		predicted m/z	obs. from $[M+17H]^{17+}$	predicted m/z	obs. from $[M+17H]^{17+}$
Sheep	3	728.0	----	909.8	yes
Beef	4	732.6	yes	915.5	yes
Horse	4	727.2	yes	908.8	yes
Pork	4	728.0	yes	909.8	yes