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POSTER

Characterization and kinetics studies of water buffalo (*Bubalus bubalis*) myoglobin

R. Dosi[1], A. Di Maro[1], A. Chambery[1], G. Colonna[2], S. Costantini[3], G. Geraci[4], A. Parente[1]

[1] Laboratorio Integrato per la Qualità e la Sicurezza degli Alimenti, CRdC "Produzioni Agroalimentari", Second University of Naples, Caserta, Italy

[2] Department of Biochemistry and Biophysics and CRISCEB, Second University of Naples, Naples, Italy

[3] Institute of Food Science, CNR, Avellino, Italy

[4] Department of Biological Sciences, Via Mezzocannone 8, University of Naples Federico II, Italy

Corresponding author: A. Parente, Laboratorio Integrato per la Qualità e la Sicurezza degli Alimenti, CRdC "Produzioni Agroalimentari", Second University of Naples, Via Vivaldi 43, I-81100 Caserta, Italy - Tel. +39 0823 274583 - Fax: +39 0823 274571 - Email: augusto.parente@unina2.it

ABSTRACT: It is generally accepted that dry-aged buffalo (*B. bubalis*) meat becomes dark faster than bovine (*B. taurus*) meat, discouraging consumer purchase. We have investigated whether this faster darkening process might depend on structural and/or kinetic differences between buffalo and bovine myoglobins (Mbs). To this end, we have purified to homogeneity buffalo Mb from *Longissimus dorsi* muscle and obtained both its Mr (17,034.5) and the complete amino acid sequence, which, compared with the bovine one, showed three amino acid substitutions: $D_{bo}141E_{bu,}$ $A_{bo}19T_{bu}$ and $A_{bo}117D_{bu.}$ As revealed by the 3D structure, they were located on the surface of the protein, far from the heme binding pocket, and did not cause appreciable structural changes. Autoxidation rates of purified buffalo and bovine myoglobins at 37 °C, pH 7.2, were almost identical (0.052±0.001 h^{-1} and 0.054±0.002 h^{-1}, respectively), as were their oxygen-binding Kd values (3.7±0.1 µM and 3.5± 0.1 µM, respectively). These data indicate that the structure of the heme pockets in the two proteins is similar, with similar functional properties. Moreover, the percent of MetMb values in the purified buffalo and bovine samples, after the same time from slaughtering, were almost identical (57% and 47%, respectively). The results presented here suggest that the faster darkening of buffalo meat depends on factors other than the oxidation rate of its Mb, as, for example, the Mb content (0.393±0.005 g/100 g) and consequently its MetMb content, which is almost twice as high as bovine meat (0.209±0.003 g/100 g), and likely other factors.

INTRODUCTION - Myoglobin (Mb) is the most important determinant for meat colour. In living animals, there is an equilibrium between the purplish-red Mb form (deoxyMb) and the cherry-red form (oxyMb or MbO_2). During meat storage, these two reduced Mb forms readily become oxidized to the brownish-red metMb (Faustman *et al*., 1990). Over the last 20 years it has been shown that there is up to a 12-fold difference in the rate at which Mb oxidizes in post-mortem mammalian muscles, depending on the species and muscle

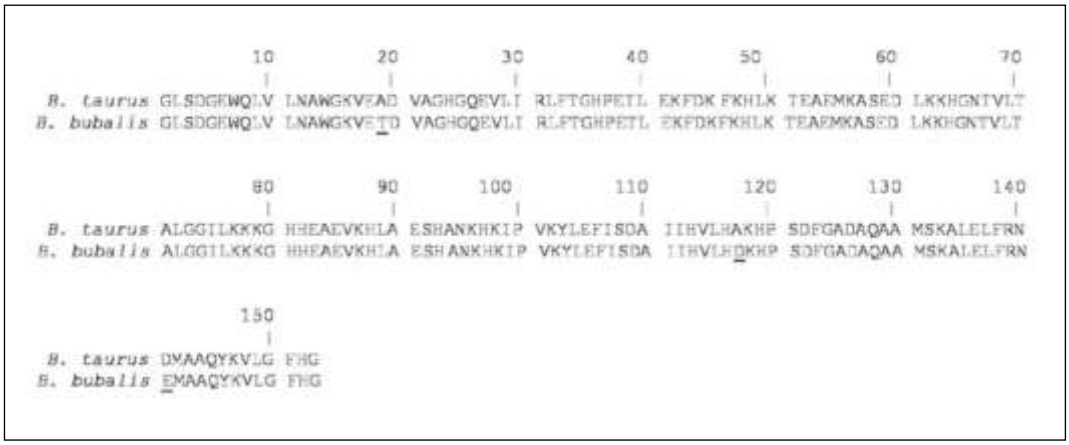
type (Livingston *et al*., 1986; Foucat *et al*., 1994; Tada *et al*., 1998; Stewart *et al.,* 2004). Recently, water buffalo meat, derived from male animals, is being introduced in the fresh meat market of Southern Italy, as an alternative to the bovine one. Compared to the latter, water buffalo meat is presented as having higher nutritional properties, although there are conflicting reports (Syed Ziauddin *et al*., 1993; Cutrignelli, 1996; Infascelli *et al*., 2004; Spanghero *et al*., 2004). The aim of this study is to investigate whether the reasons for the faster darkening process of buffalo meat might depend on structural differences between buffalo and bovine Mbs. Therefore, we have: i) purified buffalo Mb; ii) determined its average amount in meat; iii) determined its primary structure and main structural properties, and iv) studied Kd values and autoxidation kinetics to establish the possible occurrence of correlation with the amino acid substitutions.

MATERIAL AND METHODS - Myoglobin was purified from the Italian water buffalo (*Bubalus bubalis*) skeletal muscle (*Longissimus dorsi*), provided by "Cooperativa La Baronia", Pontelatone, Caserta (Italy). Buffalo Mb was purified using the following procedure: i) homogenization; ii) dialysis against MilliQ water; iii) gel-filtration on a Sephacryl S-100 column and, iv) anion-exchange chromatography on a DEAE-Sepharose column. Mb concentration was determined at the isosbestic point of 527 nm using a $E_{1\%,1cm}$ coefficient of 2.7. Determination of percent values of MetMb in the purified preparations of buffalo and bovine samples, collected after the same time from slaughtering, were performed spectroscopically according to Krzywicki (1982). The autoxidation of MbO_2 to metMb was monitored by recording, over time, the changes of the absorption spectrum in the range 500–700 nm at measured time intervals, and estimating the absorbance decrease at 582 nm. MbO_2 was prepared by sodium dithionite reduction of pure Mb. The relative molecular mass of buffalo apo-Mb, obtained from RP-HPLC in the presence of 0.1% TFA, was determined using a Q-TOF *Micro* mass spectrometer, equipped with a CapLC system. The acquisition and deconvolution of data were performed on a Mass Lynx Windows NT PC data system. Automated Edman degradation was performed on the first 41 amino acid residues of the HPLC purified buffalo apoMb. Buffalo and bovine apo-Mbs were digested with trypsin, Glu-C and cyanogen bromide (CNBr). Peptides masses were determined by MALDI-TOF mass spectrometry. Signals recorded in the mass spectra were associated with the corresponding peptides on the basis of the expected molecular mass calculated by using a suitable computer program (Peptide Tools, Hewlett-Packard). When necessary, Edman degradation steps were performed on the HPLC purified peptides, in order to confirm the assignment. The three-dimensional models of bovine and buffalo myoglobins were created by homology modelling, using the MODELER/QUANTA software.

RESULTS AND CONCLUSIONS - The purification procedure described in Materials and methods yielded reproducibly purified preparations of Mbs from the skeletal muscle of both water buffalo and bovine. The amount of Mb in samples from both animal sources was 0.393±0.005 g/100 g of tissue for buffalo and 0.209±0.003 g/100 g of tissue for bovine. Percent MetMb values in the purified buffalo and bovine samples, after the same time from slaughtering, were 57% and 47%, respectively. The combined use of mass spectrometry and automated Edman degradation for the analysis of peptides obtained from trypsin, Glu-C and CNBr digestions, allowed us to obtain the entire sequence of B. bubalis

myoglobin. Further, the agreement between the experimental mass of the native protein (17,034.5 Da) and that calculated on the basis of the amino acid sequence (17,034.46 Da) is a further confirmation of the correctness of the analysis. The amino acid sequence of buffalo Mb, compared with the bovine one, shows three amino acid substitutions (1.96%) out of 153 amino acid residues, one of which is conservative $D_{bov}141E_{buf}$ and two non-conservative, $A_{bov}19T_{buf}$ and $A_{bov}117D_{buf}$ (Fig. 1). The three-dimensional structures of bovine and buffalo Mbs, obtained by homology modelling, are very much alike and, also, the Mb amino acids at the interface with the heme are well conserved in both organisms. The three amino acid substitutions between buffalo and bovine Mbs are located on the surface of the protein and far from the heme binding pocket and do not cause appreciable structural changes. It appears unlikely that they may be responsible of changes in molecular properties. Nevertheless, helices A and G in buffalo Mb are more destabilized. This last result may be explained considering that, in buffalo, helix A, in position 19, has a threonyl amino acid residue, that is a destabilizing β-branched residue ($A_{bov}19T_{buf}$). In fact, it is known that an overall greater stability of helices in proteins is related to a combination of stabilizing factors, the most common being the low content of β-branched residues. Moreover, the presence of a negatively charged residue ($A_{bov}117D_{buf}$) at the C-terminus of helix G, certainly induces a destabilization of the helix dipole (Richardson *et al.,* 1988). Buffalo and bovine MbO2, at pH 7.2 and 37 °C, are equally resistant to autoxidation, with Kd values of 0.052±0.001 h^{-1} and 0.054±0.002 h^{-1}, respectively. The O_2 dissociation curves of purified bovine and buffalo Mbs, in identical experimental conditions at pH 7.2, were almost superimposable, providing similar Kd values (3.7±0.1 μM and 3.5±0.1 μM, respectively). These observations indicate that the similar structural architecture of the two proteins heme pockets involve similar functional properties. Therefore, Mb autoxidation rate does not influence buffalo meat colour (Dosi *et al*., 2006). This might depend on the higher Mb content found, likely together with other factors (Faustman *et al*., 1990).

Figure 1. Amino acid sequence of *Bubalus bubalis* Mb, including the peptides obtained by treatment of Mb with trypsin, Glu-C endoproteinase and cyanogen bromide, compared to *Bos taurus* Mb. Substitutions are underlined.



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