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Amino acid sequence of myoglobin from emu (*Dromaius novaehollandiae*) skeletal muscle ☆

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ABSTRACT

The objective of the present study was to characterize the primary structure of emu myoglobin (Mb). Emu Mb was isolated from *Iliofibularis* muscle employing gel-filtration chromatography. Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry was employed to determine the exact molecular mass of emu Mb in comparison with horse Mb, and Edman degradation was utilized to characterize the amino acid sequence. The molecular mass of emu Mb was 17,380 Da and was close to those reported for ratite and poultry myoglobins. Similar to myoglobins from meat-producing livestock and birds, emu Mb has 153 amino acids. Emu Mb contains 9 histidines. Proximal and distal histidines, responsible for coordinating oxygen-binding property of Mb, are conserved in emu. Emu Mb shared more than 90% homology with ratite and chicken myoglobins, whereas it demonstrated only less than 70% sequence similarity with ruminant myoglobins.

1. Introduction

Exotic meats are gaining popularity in the global meat market, where health-conscious consumers are becoming increasingly careful in choosing lean alternatives over traditional red meats (Daniel, Thompson, & Hoover, 2000; Hoffman & Wiklund, 2006). Recent reviews illustrated that exotic meats are excellent sources of dietary nutrients and have quality attributes comparable to red meats (Hoffman, 2008a; Hoffman & Wiklund, 2006; Saadoun & Cabrera, 2008). Among exotic meat species, ratites received significant attention as producers of low-fat meats (Andrews, Gillespie, & Schupp, 2000; Hoffman, 2008b; Sales & Horbanczuk, 1998). Ratites are a family of flightless birds, such as ostrich, emu, and rhea, which now are intensively farmed for their valuable products, including meat (Gillespie & Schupp, 1998). Furthermore, Hoffman (2005) argued that ratites have significant potential in the regions where consumers are seeking red meat sources free of prion diseases.

Emu (*Dromaius novaehollandiae*) is the second largest bird in the world and is the largest avian species native to Australia, where commercial emu farming has been successful since the 1980s (Sales, 2007). In the United States, more than one million emus are raised as a specialty livestock for meat, oil, and leather (American Emu Association, 2009). Furthermore, emu meat has been marketed successfully in Europe and the United States as gourmet products. The nutrient profile and quality attributes of emu meat have been investigated by several researchers, and emu meat was found to be a better source of heme iron than beef (Berge, Lepetit, Renerre, & Touraille, 1997). Consumption of emu meat is recommended by the American Heart Association because of its leanness, low cholesterol content, and a heart-favorable fatty acid profile (Beckerbauer et al., 2001; Daniel et al., 2000; Sales & Horbanczuk, 1998; Wang, Sunwoo, Sim, & Cherian, 2000). In addition, Pegg, Amarowicz, and Code (2006) concluded that emu meat, being an excellent natural source of creatine, demonstrated potential as a functional food for athletes in high endurance sports. Emus have greater muscular activity than common domesticated poultry species (Sales, 2007), and therefore emu meat is darker in color compared to poultry meat due to an increased myoglobin (Mb) content. Thus, color of emu meat is similar to red meats harvested from livestock.

Myoglobin is the sarcoplasmic heme protein primarily responsible for the red color of meat from animals and birds. In live animals, Mb serves both oxygen-storage and oxygen-delivery functions in skeletal muscles (Wittenberg & Wittenberg, 2003). Mammalian and avian myoglobins are comprised of 153 amino acids, and the primary sequence of Mb depends upon species (Livingston & Brown, 1981). Despite differences in amino acid sequence, the functional properties of this monomeric heme protein are well conserved across many species

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(Wittenberg & Wittenberg, 2003). Nevertheless, the differences in amino acid sequence of Mb are responsible for various species-specific phenomena in fresh meat color, such as lipid oxidation-induced browning in beef (Suman, Faustman, Stamer, & Liebler, 2007) and rapid discoloration of buffalo meat (Dosi et al., 2006). In the perspective of meat color, while livestock myoglobins received significant attention relative to primary structure characterization, avian myoglobins received limited attention. Recently, Enoki, Ohga, Ishidate, and Morimoto (2008) characterized the primary structure of 31 avian species and concluded that avian myoglobins are different from their mammalian, as well as livestock, counterparts with respect to amino acid sequence.

The molecular masses of avian myoglobins, in general, are 300–400 Da greater than those of livestock counterparts (http://www.expasy.org). Chicken Mb was characterized more than three decades ago (Deconinck et al., 1975), and the primary structures of myoglobins from ratites, such as ostrich and rhea, have been determined recently (Enoki et al., 2008). In our recent investigation, turkey Mb exhibited greater molecular mass than beef Mb (Joseph, Suman, Li, Beach, & Claus, 2010). A search in the post-genomic era protein databases (http://www.expasy.org; http://www.ncbi.nlm.nih.gov) revealed that the primary structure of emu Mb has not been determined. Therefore, the objective of the present study was to determine the primary structure of emu Mb.

2. Materials and methods

2.1. Materials and chemicals

Emu Mb is not commercially available and therefore was isolated from skeletal muscle. Emu fan fillets, comprised of *Iliofibularis* muscle, were procured from BackCountry Emu Products (Whitehouse, TN, USA), shipped overnight to the Meat Laboratory at the University of Kentucky, and frozen at −80 °C until use. Sephacryl 200-HR, horse Mb, ammonium sulfate, ammonium bicarbonate, Tris–HCl, EDTA, sodium hydrosulfite, glycerol, acrylamide, methanol, acetic acid, beta-mercaptoethanol, acetonitrile, trifluoroacetic acid, formic acid, sinapinic acid, and Coomassie blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). PD-10 columns were obtained from GE Healthcare (Piscataway, NJ, USA), whereas the marker proteins were purchased from Bio-Rad (Hercules, CA, USA). All chemicals were of reagent grade or greater purity.

2.2. Myoglobin isolation

Emu Mb was purified from muscle *Iliofibularis*, via ammonium sulfate precipitation and gel-filtration chromatography according to Faustman and Phillips (2001), with minor modifications. Briefly, muscle was homogenized in buffer (5 mM Tris–HCl, 1 mM EDTA, pH 8.0, 4 °C) and centrifuged at 5000 × *g* for 10 min. The supernatant was brought to 50% ammonium sulfate saturation and centrifuged at 18,000 × *g* for 20 min. The resulting supernatant was saturated with ammonium sulfate (100%) and centrifuged at 20,000 × *g* for 1 h. The precipitate was resuspended in homogenization buffer and dialyzed against 5 mM Tris–HCl, 1 mM EDTA, at pH 8.0, 4 °C for 24 h. Myoglobin was separated from hemoglobin using a Sephacryl 200-HR gel-filtration column (2.5 × 100 cm). The elution buffer, containing 5 mM Tris–HCl, 1 mM EDTA at pH 8.0, 4 °C, was used at a flow rate of 60 ml/h.

Freshly prepared emu Mb was subjected to SDS-PAGE to determine the purity of the sample as described by Laemmli (1970). Duplicate samples were prepared (2 mg/ml of protein solution) with SDS-PAGE sample buffer (4% SDS solution, 20% glycerol, 0.125 M Tris, pH 6.8, 10% beta-mercaptoethanol) at a 1:1 ratio. The stacking and resolving gels were 3% and 15% acrylamide, respectively. An aliquot of 15 μl from each sample was loaded onto the gel. The protein standard was a cocktail of 9 individual marker proteins (molecular weight 6.5 to 200 kDa). The electric current for each gel was maintained at 15 mA using a Mini Protean II unit (Bio-Rad, Hercules, CA, USA). After separation, the proteins were stained with 0.01% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol and 6.8% (v/v) acetic acid, and destained with 10% methanol (v/v) and 7.5% (v/v) acetic acid.

2.3. Determination of molecular weight of emu myoglobin

Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) was used to determine the exact molecular mass of emu Mb in comparison with horse Mb, which is a well-characterized heme protein. Before MS analysis, Mb samples were transferred to 20 mM ammonium bicarbonate buffer using PD-10 desalting columns to remove inorganic salts. Ammonium bicarbonate buffer was used to minimize interferences with mass spectrometric analysis (Manza, Stamer, Ham, Codreanu, & Liebler, 2005; Suman, Faustman, Stamer, & Liebler, 2006; Suman et al., 2007; Joseph et al., 2010). Approximately 1 μl Mb sample was mixed with 1 μl freshly prepared 50% solution of sinapinic acid (in 0.5% trifluoroacetic acid and 50% acetonitrile); 0.5 μl mixture was spotted on the MALDI target plate, scratched with pipette tip to spread uniformly and allowed to dry for 10 min. Protein molecular ions were analyzed in linear, positive ion mode using a Ciphergen PBSIIC linear MALDI-TOF mass spectrometer (Ciphergen Biosystems, Fremont, CA, USA) at a digitizer rate of 250 MHz, pulse voltage 3000 V, source voltage 20,000 V, and laser intensity 220. Lysozyme and beta-lactoglobulin were used as internal protein standards. Each spot was analyzed to accumulate spectra composing of 300 shots, with a detector voltage of 2800 V, focus mass of 16,500 Da, and deflector mass of 3000 Da. The resulting spectra were averaged, noise-smoothed, baseline-corrected, and mass-calibrated.

2.4. Determination of amino acid sequence

Purified emu Mb was treated with 0.1% SDS, and subjected to N-terminal sequencing by Edman degradation in a Procise 494 system (Applied Biosystems, Foster City, CA, USA) to determine the identity of thirty-nine residues from amino terminus, whereas the analyses of tryptic- and cyanogen bromide-peptides yielded the rest of the sequence. Prior to tryptic digestion, emu Mb was separated in SDS-PAGE gel and treated as described by Rosenfeld, Capdevielle, Guillemot, and Ferrara (1992) with the few modifications. The initial wash was increased to 1 h at 37 °C, and the gel band was dried completely using a speed-vac system. Myoglobin sample was subjected to in-gel digestion at 37 °C for 18 h at a trypsin:protein ratio of 1:20 (w/w). The resulting peptide mixture was dissolved in 2% acetonitrile/0.1% trifluoroacetic acid and separated in a reverse-phase HPLC system (Michrom BioResources, Auburn, CA, USA) using a C-18 column. The separated peptides were detected at 215 nm using a UV–VIS detector, and the molecular masses of peptides were analyzed in a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) before subjecting them to Edman degradation. Emu Mb was subjected to chemical digestion with cyanogen bromide vapor according to D'Souza, Ginsberg, Burke, and Plow (1990). Briefly, three micrograms of SDS-treated Mb was loaded on a polyvinylidene difluoride membrane using a Prosorb filter (Applied Biosystems, Foster City, CA, USA), washed with 20% acetonitrile containing 0.1% trifluoroacetic acid, and dried. The polyvinylidene difluoride membrane was exposed to the vapor from 22 mg/ml cyanogen bromide (in 70% v/v formic acid) at room temperature in darkness for 20 h. Excess cyanogen bromide was removed by aerating the polyvinylidene difluoride membrane for 15 min in a fume hood, and the peptides were subjected to Edman degradation. The primary structure of emu Mb was used to determine the sequence similarities with myoglobins from other birds and livestock (Ye, McGinnis, & Madden, 2006).

3. Results and discussion

Primary structure influences several molecular mechanisms governing myoglobin's physiological functions, such as autoxidation