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Evaluation of different methods for determination of the iron saturation level in bovine lactoferrin

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ABSTRACT

Lactoferrin (Lf) samples with ca. 25%, 50%, 75%, 85% and 100% iron saturation were prepared for the purpose of evaluating Chromametry, Differential Scanning Calorimetry (DSC) and Circular Dichroism (CD) spectropolarimetry for their suitability in determining the iron saturation level. Numerical values for colour from Chromametry, enthalpy change of denaturation (ΔH_{cal}) from DSC and molar ellipticities from CD were statistically analysed to evaluate their correlation with the level of iron saturation in Lf. Linear regression analysis of colour coordinates Chroma (C^*) and hue (h°) angle on percentage iron saturation level of Lf showed that the values can be used to estimate the iron saturation level. The ΔH_{cal} for the iron saturated peak and the CD ellipticities in the 310–340 nm region provided reliable data for the estimation of iron saturation level of Lf up to 75%. Mono- and di-saturated Lf displayed the same thermal stability and very similar tertiary structures.

1. Introduction

Lactoferrin (Lf), a glycoprotein found in different biological fluids of mammals, belongs to the transferrin family, responsible for regulation of iron level in vertebrates (Abdallah & El Hage Chahine, 2000; Conesa et al., 2008). Bovine Lf is composed of a single polypeptide chain having 689 amino acids and 4 glycans and has a molecular mass of 80,000–84,000 Daltons. Lf is folded into two homologous globular lobes, N- and C-lobes representing the N and C-terminals of the molecule, respectively. Each lobe is composed of two sub-lobes. These sub-lobes are capable of reversibly binding a ferric ion (Fe^{3+}) in the presence of (bi) carbonate (Steijns & van Hooijdonk, 2000). Lf has the ability to bind iron with high affinity ($K_D \sim 10^{-20}$ M) but can also bind many other metal ions with lower affinity (Baker & Baker, 2005; Indyk, McGrail, Watene, & Filonzi, 2007; Moore, Anderson, Groom, Haridas, & Baker, 1997). In nature, bovine Lf, exists in a partly iron-saturated (15–20%) form (native-Lf), which has been described as mixture composed of iron free (apo-) Lf, iron saturated (holo-) Lf but also monoferric Lf species saturated at either their N or C lobe (Brisson, Britten, & Pouliot, 2007).

The molecular conformation of Lf changes depending upon the binding of iron to its iron binding sites. Lf having bound iron has a more compact structure compared to iron free Lf as the open inter-domain cleft in each lobe of the Lf, are closed over in the case where the iron ion is bound. Thus, the compact molecular structure of iron saturated Lf has been attributed to its higher resistance to thermal denaturation and proteolysis than iron free Lf (Sánchez et al., 1992). With regards to the lobe structure, the two domains of the C-lobe are more closed over the iron site than those of the N-lobe, which lead to greater thermodynamic stability of, and slower iron release from the C-lobe compared with the N-lobe (Anderson, Baker, Norris, Rice, & Baker, 1989). Apart from the closed lobe structure over iron, the affinity for iron of the C-site has been reported to be higher than that of the N-site (Abdallah & El Hage Chahine, 2000).

The percentage iron saturation of Lf is most commonly determined from UV–Visible spectrophotometry by measuring the ratio of A_{465nm}/A_{280nm} (Brisson et al., 2007; Hashizume, Kuroda, & Murakami, 1987; Ainscough et al., 1980; Hadden, Bloemendal, Haris, Srai, & Chapman, 1994; Mazurier & Spik, 1980). Due to the large difference in absorbance values at the two wavelengths used, however, large errors can easily be introduced using this method as we have previously reported (Bokkhim, Bansal, GrØndahl, & Bhandari, 2013). Very recently, Majka et al. (2013) also reported that spectrophotometric method can lead to substantial deviations when used to measure the iron content in lactoferrin samples containing a mixture of diferric and monoferric forms. Alternatively,

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methods which measure the total amount of iron directly have been reported and include Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES) (Bokkhim et al., 2013; Sreedhara et al., 2010); Atomic Absorption Spectrophotometer (AAS) (Paulsson, Svensson, Kishore, & Naidu, 1993) and radioactivity (Bovell-Benjamin, Viteri, & Allen, 2000). Such approaches are valid when it can be shown independently that all the iron present in a sample is bound to the protein. In combination with iron determination, the Dumas method is typically used to determine the protein content in Lf (Bokkhim et al., 2013; Brisson et al., 2007) thereby allowing calculation of the iron saturation level.

The colour of Lf is due to the presence of bound iron and its intensity depends upon the degree of iron saturation (Steijns & van Hooijdonk, 2000). This forms the basis of the use of UV–visible spectroscopy to determine iron saturation and has been recently quantified using a Chromameter by Bokkhim et al. (2013) in terms of the colour space parameters and polar coordinates. The study showed that redness, yellowness and colour intensity increased with increasing level of iron saturation while whiteness decreased and the colour perception shifted from yellow towards red.

Differential Scanning Calorimetry (DSC) has been used widely to detect and monitor the thermally induced conformational and phase transitions in samples as a function of temperature. DSC has been widely applied to the study of the thermal resistance of Lf to denaturation which has been found to depend on the form of Lf (Bengoechea, Peinado, & McClements, 2011; Bokkhim et al., 2013; Paulsson et al., 1993; Rüegg, Moor, & Blanc, 1977; Sánchez et al., 1992). Thus, iron free (apo-), partially iron saturated (native-) and iron saturated (holo-) -Lf demonstrated different denaturation rates and temperatures. Most studies have reported one thermal transition for apo-Lf and holo-Lf but two for native-Lf (Bengoechea et al., 2011; Bokkhim et al., 2013; Paulsson et al., 1993; Sánchez et al., 1992).

Circular Dichroism (CD) spectropolarimetry has been used extensively to characterise the secondary and tertiary structure of proteins in solution by measuring absorption of left and right circularly polarised light (Yang, Wu, & Martinez, 1986). For Lf, the CD spectra reported in a number of studies have been in good agreement (Brown & Parry, 1974; Mazurier, Aubert, Loucheux-Lefevre, & Spik, 1976; Nam, Shimazaki, Kumura, Lee, & Yu, 1999; Shimazaki, Kawano, & Yoo, 1991) and have shown that iron binding of Lf only affected the tertiary structure leaving the secondary structure unaltered (Bokkhim et al., 2013; Brown & Parry, 1974). Furthermore, Shimazaki et al. (1991) concluded that the CD spectrum of native-Lf is composed of the combined CD bands of apo- and holo-Lf.

Researches in the past have focussed on the characterisation of the three most common forms of Lf (apo-, native- and holo-Lf) but have not systematically studied the effect of increasing levels of iron saturation on parameters mentioned above; colour, thermal stability and CD spectra. Studies on the effect of the level of iron saturation on these parameters will enable the development of a simple and reliable instrumental method to determine the level of iron saturation in Lf samples.

This study aimed to investigate the potential of DSC, CD and colorimetric techniques for the evaluation of the level of iron saturation in Lf and also to provide further insight into the mechanism of iron binding by Lf. The experiments were performed on the iron free or apo- (0.9%), native- (12.9%), partly iron saturated Lf, ca. 25% (Lf-25), ca. 50% (Lf-50), ca. 75% (Lf-75), ca. 85% (Lf-85) and completely iron saturated (holo-) Lf. A correlation between the percentage of iron saturation and denaturation enthalpy, colour intensity and molar ellipticity was established. Furthermore, the data obtained enabled evaluation of the mechanism for iron binding to Lf.

2. Materials and methods

2.1. Materials

Bovine lactoferrin (NatraFerrin) in its iron free state (~0.9%) and native form (partially iron saturated, ~13%) with purity of 97% and 96.3% (of protein content) respectively were provided by MG Nutritionals®, Australia. Tris (hydroxymethyl) methane and potassium chloride (purity > 99.8%); sodium bicarbonate (purity > 99.7%); sodium acetate trihydrate, nitrilotriacetic acid (NTA), sodium hydroxide and acetic acid (purity > 99%); ferric nitrate nonahydrate and sodium chloride (purity > 98%) and hydrochloric acid (concentration > 31.5%) were purchased from one of the following companies: Merck Pty. Ltd., Biolab (Aust) Ltd., Chem-supply Pty. Ltd., Ajax Finechem Pty. Ltd. and Sigma Aldrich Co., Australia. Cellulose membrane dialysis tubing with an average flat width of 76 mm (D9402) and a 12,400 Dalton molecular mass cut off was purchased from Sigma Aldrich Co., Australia. All chemicals used in this study were of analytical grade. Millipore water was used for the preparation of all solutions.

2.2. Preparation of iron saturated Lf

Lactoferrin samples with different levels of iron saturation were prepared from native-Lf following the procedure described in our previous paper (Bokkhim et al., 2013). Calculated amounts of freshly prepared ferric nitrilotriacetic acid (FeNTA) solution [9.9 mM ferric nitrate and 8.5 mM nitrilotriacetic acid (disodium salt) in milliQ water, pH adjusted to 7.0 with solid sodium bicarbonate] was added to the Lf solution to achieve the calculated molar ratio of iron:Lf of 2:1, 1.7:1, 1.5:1, 1:1 and 0.5:1 respectively. The mixtures were incubated at room temperature (~20 °C) for 1 h, dialysed against Millipore water for 48 h under constant stirring with at least three changes of water and finally freeze dried. Gel electrophoresis (SDS PAGE under reducing conditions) was run to verify the purity of the Lf (commercial & dialysed) samples.

2.3. Measurement of iron saturation in Lf

To determine the iron saturation level in freshly prepared Lf, the total iron content of the Lf samples was measured by Inductively Coupled Plasma Optical Emission Spectroscopy (Varian Vista Pro Radial ICP-OES system, Melbourne, Australia) and the protein content by Dumas method (Rayment & Higginso, 1992). The values of iron content (mg/L) obtained from ICP-OES were converted to percentage iron saturation based on the protein content (Lf) to iron. Based on 2 mol of iron per mole of Lf, a theoretical value of 1.4 mg iron per g Lf is considered as 100% saturation.

2.4. Measurement of colour of Lf samples

A hand-held Minolta Chromameter CR-400 (Japan) was used to measure the colour of Lf in aqueous solution (1% wt/wt). The L^*, a^*, b^* colour space parameters developed by Commission International de l'Éclairage (CIELAB) were used to evaluate the differences in colour. The colour space parameters are calculated from measured reflectance spectra representing red (R), green (G) and blue (B) regions in the visible wavelength region 400–700 nm. The calculated colour space values enable the colour to be defined in terms of its Cartesian coordinates with respect to the axes L^*, a^* and b^* in a uniform three dimensional colour space. The L^* value represents lightness, or a spectrum from black = 0 to white = 100, a^* represents a spectrum from red (+) to green (−) and b^* represents a spectrum from blue (−) to yellow (+). However, the values of a^* and b^* need to be converted into polar coordinates, Hue angle