Biometals (2012) 25:1083–1088
DOI 10.1007/s10534-012-9573-3

Iron-dependent binding of bovine milk α-casein with holo-lactoferrin, but not holo-transferrin

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Received: 28 March 2012 / Accepted: 10 July 2012 / Published online: 24 July 2012

Abstract Bovine milk α-casein has been identified as an iron- and heme-binding protein. However, the physiological role of its iron-binding remains to be elucidated in more detail. α-Casein was immobilized on CNBr-activated Sepharose 4B beads, and the α-casein agarose beads efficiently bound hemin as well as ferrous ammonium sulfate (Fe^{2+}) as compared with control beads. Additionally, α-casein-beads bound bovine holo-lactoferrin (Lf), but not holo-transferrin. Lf caused the release of Fe^{2+} which had bound to the α-casein–agarose beads beforehand. These results suggest that bovine α-casein iron-dependently binds holo-bovine Lf more strongly than Fe^{2+}, and that strong binding between them may play a physiological role in regulating iron homeostasis in the bovine mammary gland.

Keywords α-Casein · Holo-lactoferrin · Holo-transferrin · Iron · Milk

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Introduction

Bovine milk proteins are among the most important food proteins consumed and utilized in terms of nutritional value and functional characteristics (Farrell et al. 2004; Hurrell et al. 1989; Madureira et al. 2007). There are two major categories of milk protein that are widely defined by their chemical composition and physical properties (Farrell et al. 2004). The casein family is defined by phosphoproteins and coagulates or precipitates by acidification to pH 4.6 (Farrell et al. 2004; McMahon and Oommen 2008). The other category, whey (serum) proteins, have been used to describe a group of milk proteins that remain soluble in milk after the acid precipitation of the casein proteins, and that have no phosphorus (Farrell et al. 2004). In bovine milk, approximately 82 % of milk protein is casein, and the remaining 18 % is whey (serum) protein (Farrell et al. 2004; Swaisgood 1995; Madureria et al. 2007). Bovine casein contains 4 caseins, 2 alpha (α_{s1}- and α_{s2}-caseins), 1 beta (β-casein), and 1 kappa (κ-casein) (Farrell et al. 2004; McMahon and Oommen 2008). The caseins in milk form complexes called micelles that consist of different subunits of caseins (α_{s1}-, α_{s2}-, and β-caseins) held together by calcium phosphate due to their high phosphate content, and κ-casein helps to stabilize the micelle in solution (Farrell et al. 2004). Casein micelles are calcium phosphate nanoclusters held together by phophoryserine domains of α_{s1}-, α_{s2}- or β-casein, or a combination, resulting in the orientation

of their hydrophobic domains outward followed by their binding to other casein molecules (Farrell et al. 2004; McMahon and Oommen 2008). Casein micelles interact with Zn^{2+} and $Fe^{2+/3+}$ as well as Ca^{2+} (McMahon and Oommen 2008; Reddy and Mahoney 1991; Singh et al. 1989; Usami et al. 2011), and chelated Fe^{3+} to the calcium casein micelles has been observed in fluid milk despite a high concentration of free calcium ions (Hegenauer et al. 1979). These metal ion-binding properties of casein may affect the bioavailability of these elements for infants (Lönnerdal et al. 1982; Hurrell et al. 1989).

Lactoferrin (Lf) is an 80 kDa iron-binding glycoprotein in the same family as serum transferrin. The milk Lf concentration in normal lactating cows ranges from 1.15 to 485.3 μg ml^{-1} (Adlerova et al. 2008). Its concentration markedly increases (even to 100 mg ml^{-1}) during mammary gland involution (Adlerova et al. 2008), and it is known to form high-weight molecular complexes (Wang and Hurley 1996). Recently, casein micelles have been revealed to bind Lf, and the Lf-casein complexes are stable and disintegrate slowly (Anema and de Kruif 2011).

Heme-mediated binding of α-casein to ferritin and its preferential Fe^{2+}-binding have recently been reported, suggesting that α-casein can be identified as an iron- and heme-binding protein (Usami et al. 2011). In this study, we examined its ability to bind iron- and heme-binding proteins using α-casein–Sepharose 4B beads. We also demonstrated its binding mechanism with holo-Lf.

Chemicals

Bovine milk α-casein (≥70 % α_S-casein, 90 % purity by electrophoretic data), bovine Lf and apo-transferrin (Tf), hemin (ferriprotoporphyrin IX chloride), and ferrozine were purchased from Sigma (St. Louis, MO, USA). CNBr-activated Sepharose 4B and Sepharose 4B were purchased from GE Healthcare (Pickaway, OH, USA). One-Step™ Turbo TMB (3,3′,5,5′ tetramethylbenzidine)-ELISA and Immuno Plate Maxisorp F96 microplates were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Assay microplates for iron and hemin measurements were purchased from Iwaki Brand Div., Asahi Techno Glass (Funabashi, Chiba, Japan). Microcon YM-30 (molecular weight cutoff: 30,000) was purchased from Millipore Corp. (Billerica, MA, USA). Other chemical compounds of analytical grade were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Pure water (Elix water) was obtained using Millipore's Elix Advantage Water Purification System (Billerica, MA, USA) from tap water.

Iron and hemin measurements

Iron was measured spectrophotometrically with a microtiter plate by using ferrozine reagent (Stookey 1970). Ferrous ammonium sulfate (FAS) was freshly dissolved with Elix water, and 75-μl aliquots of FAS or sample solution was added to the wells of a microtiter plate followed by the addition of 75-μl aliquots of the reducing and iron-releasing reagent (2 M thioglycolic acid, 2 M HCl, and 10 % (w/w) trichloroacetic acid), and 75-μl aliquots of neutralizing reagent of 30 % (w/w) sodium acetate. Aliquots of 7.5 μl of 10 mM ferrozine solution as a chromogen reagent were added to each well. After incubation for 30 min, the absorbance of the mixture was measured by using a spectrophotometer at a wavelength of 562 nm.

A solution of hemin was freshly prepared by dissolving 5 mg of hemin in a minimum amount of 0.1 M NaOH. The hemin concentration was determined using its molar extinction coefficient at 385 nm ($\varepsilon_{385nm} = 58{,}400\ M^{-1}\ cm^{-1}$) (Seki et al. 2008). Hemin was measured spectrophotometrically as follows: aliquots of 100-μl of hemin or sample solution were added to the wells of microtiter plates followed by the addition of 100-μl TMB solution. After incubation for 30 min, 50 μl of 2 M sulfuric acid as a stop solution was added to each well. After incubation for 30 min, the absorbance of the mixture was measured by using a spectrophotometer at a wavelength of 450 nm.

Preparation of holo-Lf and holo-Tf

Commercial bovine Lf and apo-Tf were dissolved in phosphate-buffered saline (PBS: 150 mM NaCl, 20 mM sodium phosphate, pH 7.2), and then saturated with Fe^{3+} by the addition of aliquots of a solution of consisting of 10 mM $Fe(NH_4)(SO_4)_2$, 100 mM sodium citrate, and 200 mM hydrogen sodium bicarbonate (molar ratio of Fe^{3+} to Lf or Tf: 3.2). The mixture was incubated overnight at 4 °C, and was dialyzed against PBS to remove the remaining iron ions.

Binding of α-casein–Sepharose 4B to Fe^{2+} and hemin

Bovine α-casein–Sepharose 4B was prepared by coupling 50 mg of α-casein to 10 ml of CNBr-activated Sepharose 4B according to the manufacturer's instructions. α-Casein–Sepharose 4B was suspended with PBS or Elix water to 50 % (v/v). In the binding test of α-casein–Sepharose 4B to Fe^{2+}, FAS as a source of Fe^{2+} was dissolved in Elix water. FAS solution was adjusted to a final concentration of 20 μM in 0.5 ml of Elix water containing 60 μl of 50 % (v/v) α-casein–Sepharose 4B or Sepharose in Elix Water (net 30 μl beads each). After mixing on a rotator overnight at 4 °C, the suspension was centrifuged at 10,000×*g* for 5 min. The resulting supernatant was subjected to iron measurement.

Hemin solution was also freshly prepared, and hemin was adjusted to a final concentration of 20 μM in 0.5 ml of PBS solution containing 60 μl 50 % (v/v) α-casein–Sepharose 4B in PBS. After mixing on a rotator overnight at 4 °C, the suspension was centrifuged at 10,000×*g* for 5 min. The resulting supernatant was subjected to hemin measurement.

Binding test of α-casein–Sepharose 4B to holo-Lf and holo-Tf

A 500 μl aliquot consisting of PBS solution containing 0.5 mg ml^{-1} holo-Lf or holo-Tf and 30 μl of α-casein–Sepharose 4B or Sepharose 4B beads was rotated overnight at 4 °C, and then the suspension was centrifuged at 10,000×*g* for 5 min. The resulting supernatant was measured using a spectrophotometer at a wavelength of 280 nm (Fig. 2a). The photograph shown in Fig. 2b is of 5 mg ml^{-1} holo-LF and 150 μl of α-casein–Sepharose 4B or Sepharose 4B beads using the same method as described above.

Iron-dependent binding of α-casein–Sepharose 4B to holo-Lf

A 500-μl aliquot consisting of water solution containing 30 μM FAS and 40 μl of α-casein–Sepharose 4B or Sepharose 4B beads, was rotated overnight at 4 °C, and then the suspension was centrifuged at 10,000×*g* for 5 min. The resulting supernatant was discarded, and 1 ml of water solution was added and mixed, and the mixture was centrifuged under the same conditions. The resulting supernatant was discarded, and a 500 μl of PBS solution containing 0.5 mg ml^{-1} holo-Lf was added to the corresponding beads. After mixing on a rotator overnight at 4 °C, the mixture was centrifuged at 10,000×*g* for 5 min. The resulting supernatant was centrifuged at 14,000×*g* for 12 min using a Microcon YM-30 membrane with cut-off of 30,000. The filtrate solution was subjected to iron measurement for detecting iron released from the beads.

Statistical analysis

All data are expressed as the mean ± SD of four measurements. Student's *t* tests were used to compare the values obtained from the two groups. A *P* value below 0.001 was considered statistically significant.

Results and discussion

Binding of α-casein–Sepharose 4B to iron and hemin

First, we performed a binding test of α-casein–Sepharose 4B to iron and heme. α-Casein–Sepharose 4B bound hemin as well as Fe^{2+} released from FAS (Fig. 1). Although trace amounts of Fe^{2+} bound to the control beads (Sepharose 4B), most Fe^{2+} bound to α-casein–Sepharose 4B. Hemin showed weaker binding to α-casein than Fe^{2+}, and some hemin showed non-specific binding with the control beads. However, based on preliminary data, we concluded that α-casein–Sepharose 4B is applicable to detect and purify iron- and hemin-binding protein.

Iron-dependent binding of α-casein with holo-Lf

Casein micelles have been revealed to interact with Lf (Anema and de Kruif 2011). In this study, α-casein–Sepharose 4B bound holo-Lf, but not holo-Tf (Fig. 2a). Eventually, both holo-Lf and holo-Tf turned pink after saturation with 10 mM $Fe(NH_4)(SO_4)_2$, 100 mM sodium citrate, and 200 mM hydrogen sodium bicarbonate (data not shown). Neither holo-Lf nor holo-Tf released iron from their holo-types after incubation with α-casein beads, because changes in the pink color and absorbance at 280 nm were not observed in holo-Tf, and changes in the pink color

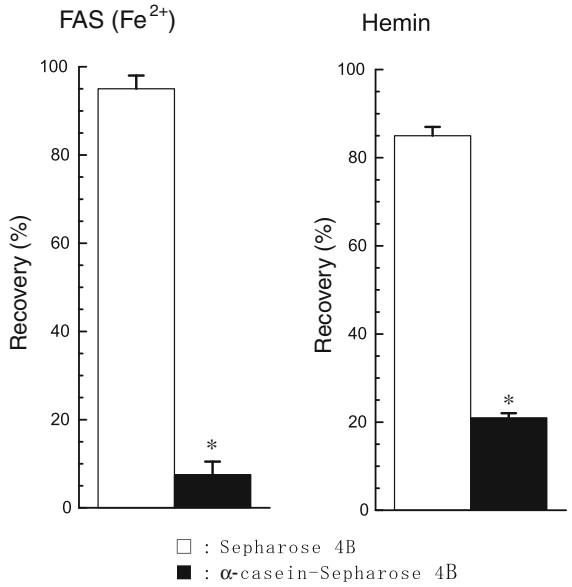

Fig. 1 Binding of α-casein–Sepharose 4B to Fe^{2+} and hemin. A 500 µl aliquot consisting of water and PBS solution containing 20 µM FAS and 20 µM hemin, respectively, and 30 µl of α-casein–Sepharose 4B or Sepharose 4B beads, was rotated overnight at 4 °C. After rotation, the mixture was centrifuged at 10,000×*g* for 5 min. The resulting supernatant was subjected for iron or hemin measurement as described in the "Materials and Methods". Recovery of Fe^{2+} or hemin was normalized to the control (100 %) in the absence of beads. Each value is the mean ± SD of four replicates. $*P < 0.001$, compared with the control beads

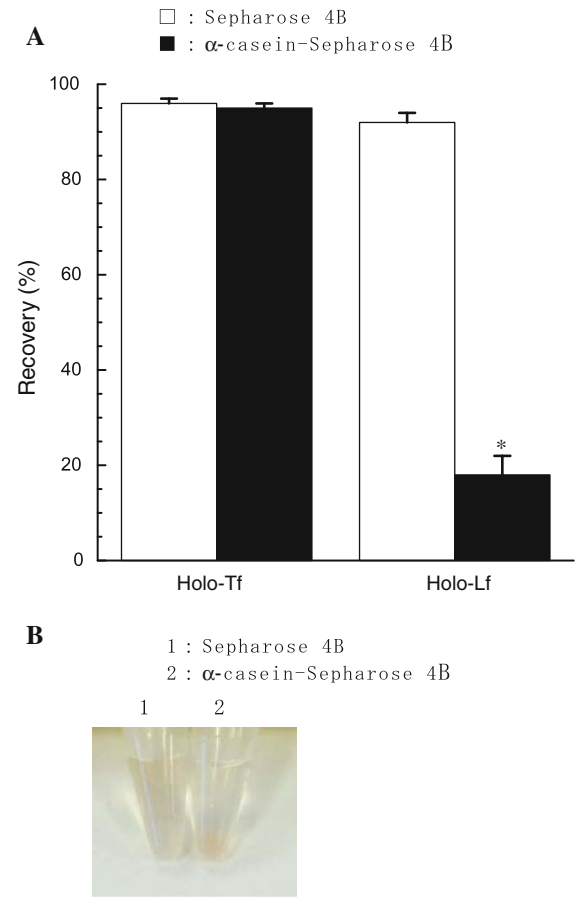

Fig. 2 The binding of α-casein–Sepharose 4B to holo-Lf and holo-Tf. **a** A 500 µl aliquot of PBS solution containing 0.5 mg ml^{-1} holo-Lf or holo Tf and 40 µl of α-casein–Sepharose or Sepharose 4B beads, was rotated overnight at 4 °C. After rotation, the mixture was centrifuged at 10,000×*g* for 5 min. The resulting supernatant was spectrophotometrically measured at an absorbance of 280 nm. Recovery of holo-Tf or holo-Lf was normalized to the control (100 %) in the absence of beads. Each value is the mean ± SD of four replicates. $*P < 0.001$, compared with the control beads. **b** A 500 µl aliquot of PBS solution containing 5 mg ml^{-1} holo-Lf and 150 µl of α-casein–Sepharose or Sepharose 4B beads was rotated overnight at 4 °C. After rotation, the photograph was taken after centrifugation of the mixture at 10,000×*g* for 5 min

of α-casein-Lf beads was not observed. Figure 2b also shows specific binding of 5 mg ml^{-1} holo-Lf to α-casein–Sepharose 4B as deduced from the change in color of α-casein-beads after centrifugation. Human Lf shows 60 % amino acid identity with human Tf (Metz-Boutigue et al. 1984), and bovine Lf has about 70 % amino acid identity with bovine Tf with microheterogeneity (Mead and Tweedie 1990; Accession number: N_M177484). Anema and de Kruif (2012) recently reported a strong ion-binding between casein family (pI = 4.6–4.8) and Lf (pI = 8.9), with higher pI value than Tf (pI = 5.5) (Legendre et al. 1985), at neutral pH. However, although commercial Lf contains holo- and apo-Lf, after binding of α-casein-beads with commercial Lf, Lf, probably apo-Lf, still remained approximately 50 % in the supernatant (data not shown). This result suggested the binding between α-casein and holo Lf is iron-mediated binding as well as ion binding under experimental condition used in this study. The present study also examined the binding affinity between Lf and α-casein. After binding of α-casein–Sepharose 4B beads with Fe^{2+}, the iron trapped α-casein-beads were incubated with holo-Lf. Figure 3 shows that the filtrate solution contained iron released from holo-Lf after trapping holo-Lf in the YM-30 reservoir as control beads did not bind iron, and the reservoir itself absorbed 10–20 % of the iron (data not shown). However,

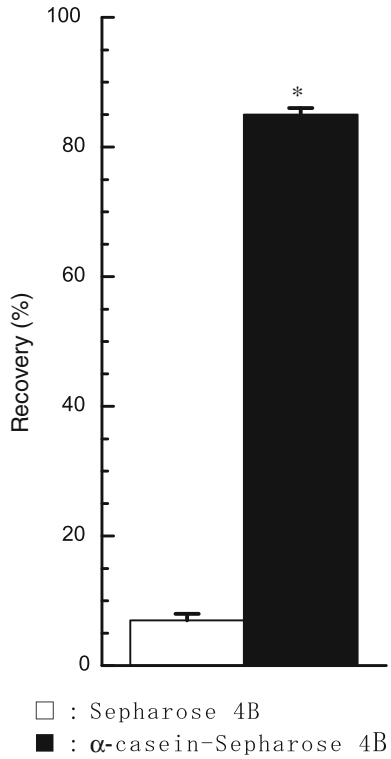

Fig. 3 Effect of holo-Lf on the binding of Fe^{2+} to α-casein-beads. A 500 µl aliquot consisting of water solution containing 30 µM FAS and 40 µl of α-casein–Sepharose 4B or Sepharose 4B beads was rotated overnight at 4 °C. After centrifugation, the resulting supernatant was discarded, and 1 ml of water solution was added and mixed, and the mixture was re-centrifuged at the same speed. The resulting supernatant was discarded, and a 500 µl aliquot of PBS solution containing 0.5 mg ml^{-1} holo-Lf was added to the beads. After rotation, the mixture was centrifuged at 10,000×*g* for 5 min. The resulting supernatant was centrifuged at 14,000×*g* for 12 min using a Microcon YM-30 (molecular weight cutoff: 30,000). The filtrate solution was subjected to iron measurement as described in the "Materials and Methods". Recovery of iron was normalized to the control (100 %) of filtrate solution of iron ions added in the absence of beads. Each value is the mean ± SD of four replicates. *$P < 0.001$, compared with the control beads

holo-Lf released most of the iron from the α-casein-iron complex because beads turned to be pink, suggesting that holo-Lf has higher affinity for α-casein than iron ions. The binding mechanism of bovine holo-Lf with α-casein may be iron-dependent, and these bindings showed higher affinity for iron binding to α-casein. Additionally, a strong electrostatic binding between α-casein and holo-Lf may also be involved in release of iron ions bound to α-casein (Anema and de Kruif 2012).

Strong interactions between α-casein and holo-Lf may provide new insight into the physiological meaning of their binding. The total iron-binding capacity of Lf (200 µg ml^{-1}) in milk from dairy cows is 2.5 µM as assumed for the apo type (Kawai et al. 1999). Bovine milk is expected to contain about 7 µM iron (Yildiz and Kayagusuzoglu 2005). Alternative iron-binding substances may be required to be present along with apo-LF. Free iron (Fe^{2+}) may cause oxidative stress mediated by the Fenton reaction (Zunquin et al. 2006). Therefore, α-casein may play an important role for protection against bacterial growth and oxidative stress by sequestering iron and heme (Orino et al. 2006; Zunquin et al. 2006). Moreover, α-casein is likely to play a physiological role to sequester the iron of saturated holo-Lf. Further studies are needed to test these hypotheses.

Acknowledgment This study was partially supported by a grant of The Morinaga Foundation for Health and Nutrition.

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