BioMetals **13:** 319–324, 2000.

Biochemical properties of canine serum ferritin: iron content and nonbinding to concanavalin A

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Received 12 September 2000; accepted 17 October 2000

Key words: canine, concanavalin A, ferritin-binding protein, ferritin iron, G subunit, serum ferritin

Abstract

A sandwich enzyme-linked immunosorbent assay using H-subunit-rich canine heart ferritin as a standard has been developed for measuring canine serum ferritin which is H-subunit-rich. Serum ferritin concentrations in 51 normal dogs ranged from 143 to 1766 ng ml^{-1}, with a mean value of 479 $\pm$ 286 (SD) ng ml^{-1}. Serum ferritin iron concentrations as determined by an immunoprecipitation technique ranged from 30.4 to 115.9 ng ml^{-1} in 15 normal dogs with serum ferritin protein levels of 298 to 959 ng ml^{-1}. There was a significant linear correlation between the serum ferritin iron and protein levels (r=0.9441, P<0.001), and the mean iron/protein ratio of serum ferritin was 0.112 ± 0.017. When canine sera were incubated with concanavalin A-Sepharose 4B, we observed the apparent binding of serum ferritin to concanavalin A. However, ferritin obtained by heat-treating the sera at pH 4.8 to remove the ferritin-binding proteins did not bind to the lectin. These results suggest that canine serum ferritin contains a considerable amount of iron but no concanavalin A-binding G subunit present in human serum ferritin.

Introduction

Ferritin, an intracellular iron-storage protein, is composed of 24 subunits of two types, termed H and L, the molecular weights of which are 21 kDa and 20 kDa, respectively (Theil 1987). Ferritin in the heart, red blood cells, and HeLa cells is H-subunit-rich, whereas it is L-subunit-rich in the liver and spleen (Arosio *et al.* 1978; Harrison & Arosio 1996).

A relatively low level of ferritin (<1 μg ml^{-1}) is present in normal human, equine, bovine, porcine, canine, and feline sera (Addison *et al.* 1972; Walters *et al.* 1973; Smith *et al.* 1984a, 1984b; Miyata *et al.* 1987; Andrews *et al.* 1992, 1994). Some of these mammalian serum ferritins have been biochemically characterized. Human ferritin purified from the serum of patients with idiopathic hemochromatosis is mainly composed of the L subunit and the glycosylated (G) subunit. The G subunit has a molecular weight of 23 kDa, binds to concanavalin A (ConA), and is immunologically similar to the L subunit (Worwood *et al.* 1979; Cragg *et al.* 1981; Santambrogio *et al.* 1987). The L subunit itself is predominant in fetal bovine serum ferritin (H/L ratio: 0.03–0.27), but the G subunit is absent (Kakuta *et al.* 1997). Human serum ferritin has a relatively low iron content (iron/protein ratio: 0.02–0.07) (Worwood *et al.* 1976; Cragg *et al.* 1981; Pootrakul *et al.* 1988), whereas fetal bovine serum ferritin has a relatively high iron content (iron/protein ratio: 0.20) (Kakuta *et al.* 1997).

We recently found that the H subunit was predominant in canine serum ferritin (Watanabe *et al.* 2000). However, other biochemical properties of ferritin remain to be elucidated. In the present study, the iron content of canine serum ferritin was measured, and its binding to ConA was examined.

Materials and methods

Blood and tissues

Blood samples were collected from randomly-selected healthy dogs and humans, and hearts and livers were obtained from dogs under pentobarbital-induced anesthesia. The serum samples and tissues were stored at −25 °C until use.

Purification of ferritin from canine heart and liver

Ferritin was purified from canine heart tissue using the procedure described for purification of bovine heart ferritin (Kakuta *et al.* 1997), except that Pefabloc SC (Merck, Darmstadt, Germany) was used in place of phenylmethylsulfonyl fluoride as a serine proteinase inhibitor. Canine liver ferritin was purified as previously described (Watanabe *et al.* 2000).

Antibodies

Antibodies to rat liver ferritin were purified from rabbit antisera by affinity chromatography as described previously (Watanabe *et al.* 2000).

Protein determination

Protein was determined according to the method of Lowry *et al.* (1951) with bovine serum albumin as a protein standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Schägger and von Jagow (1987), using 4.5% polyacrylamide stacking gel and 10% polyacrylamide running gel. Densitometry of ferritin subunit bands stained with Coomassie Brilliant Blue R250 was scanned at 565 nm using a Flying Spot Scanner (Shimadzu Model CS-9000) (Shimadzu, Kyoto, Japan).

Sandwich enzyme-linked immunosorbent assay (ELISA)

Ferritin in canine sera was determined by a sandwich ELISA using rabbit anti- rat liver ferritin antibody as described previously (Watanabe *et al.* 2000) except that canine heart ferritin instead of canine liver ferritin was used as a standard.

Human serum ferritin was measured by the following procedure. First, 100 μl of 288 ng ml^{-1} rabbit anti-human ferritin antibody (Dako, Glostrup, Denmark) in phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) was added to each well of the Immuno Plate Maxisorp F96 microtiter plates (Nunc, Roskilde, Denmark), and stored overnight at 4 °C. The antibody-coated plates were then washed with PBS containing 0.05% Tween 20 (PBST), and masked by incubation with ELISA buffer (PBS containing 0.1% gelatin and 0.1% Tween 20). Thereafter, a 100 μl aliquot of human serum or purified human spleen ferritin (Sigma, St. Louis, MO, USA) as a standard diluted with the ELISA buffer was added to each well and the plates were incubated at 37 °C for 2 h. After the plates were washed with PBST, a 100 μl aliquot of goat anti-human liver ferritin antibody (OEM Concepts, Toms River, NJ, USA), which had been appropriately diluted with ELISA buffer, was added and the plates incubated at 37 °C for 2 h. After washing, a 100 μl aliquot of alkaline phosphatase-labeled rabbit antibody against goat IgG (Fc) (American Qualex, San Clemente, CA, USA), which had been appropriately diluted with ELISA buffer, was added and the plates incubated at 37 °C for 2 h. After washing, the enzyme reaction was performed as in the previously described ELISA (Orino *et al.* 1993).

Heat-treatment of sera at pH 4.8

The serum was diluted with an equal volume of 50 mM sodium acetate buffer, pH 4.5, and titrated to pH 4.8 with 2 M acetic acid. It was then heated at 73 °C for 13 min, chilled on ice, and centrifuged at 16 000 × g for 30 min. The pH of the clear supernatant obtained was adjusted to 7.2 with 2 M Tris, and subjected to the following experiments. The percentage of remaining canine serum protein and ferritin relative to the untreated serum was 3.1 ± 0.6% (mean ± SD, n=8) and 103.1 ± 10.8%, respectively.

Determination of serum ferritin iron

Five micrograms of purified rabbit antibodies to rat liver ferritin were added to 1 ml of the ferritin solution obtained by heat-treating canine serum at pH 4.8 as described above, and the mixture was incubated overnight at 4 °C. Normal rabbit IgG (Sigma) instead of the antibody specific for rat liver ferritin was used as a control. Next, 20 μl of goat antiserum to rabbit IgG-Fc fragment (Bethyl Laboratories, Montgomery, TX, USA) was added to the mixture, and incubated

overnight at 4 °C. The mixture was centrifuged at 16 000 × *g* for 15 min at 4 °C. The resulting pellet was washed twice with 20 mM sodium bicarbonate, pH 8.2, containing 150 mM NaCl and centrifuged each time under the same condition. Two hundred microliters of 3 M HNO_3 was added to the pellet, and incubated overnight at 75 °C to dissolve the material. Iron in the nitric acid digest was assayed directly using an Atomic Absorption Spectrophotometer (Shimadzu Model AA-650) with a graphite furnace atomizer (Shimadzu Model GFA-2), under an argon atmosphere. Absorbance was measured at 248.3 nm.

Binding test of serum ferritin to ConA

Binding of ferritin in untreated canine serum to ConA was measured according to the method described by Worwood *et al.* (1979) using ConA-Sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) with minor modifications. In brief, 0.1 ml of serum was added to 0.8 ml of 50% (v/v) ConA-Sepharose 4B in 20 mM sodium phosphate buffer, pH 7.2, containing 500 mM NaCl and the total volume was made up to 1.0 ml with the same buffer. Sepharose 4B (Amersham Pharmacia Biotech AB) instead of ConA-Sepharose 4B was used as a reference. After mixing on a rotator overnight at 4 °C, the suspension was centrifuged at 420 × *g* for 5 min. The ferritin concentration in the resulting supernatant was determined by ELISA as described above.

Binding to ConA of ferritin in the solution which had been obtained by heat- treating canine or human serum at pH 4.8 as described above was also measured by the same method, except that 0.2 ml of 50% (v/v) ConA-Sepharose 4B or Sepharose 4B was added to 1 ml of the ferritin solution.

Statistical analysis

Regression analysis was used to examine the relationship between ferritin protein and ferritin iron in canine serum.

Results

Immunological difference between canine heart and liver ferritins

Figure 1 shows the SDS-PAGE pattern of purified canine heart and liver ferritins. The molecular weights of the H and L subunits of both heart and liver ferritins were 21.1 kDa and 18.7 kDa, respectively. The H/L subunit ratios of heart and liver ferritins were 3.69 and 0.43, respectively, by densitometry.

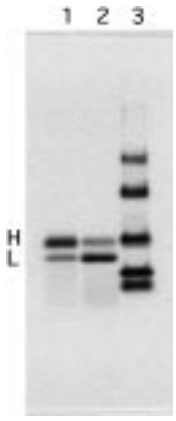

Figure 1. SDS-PAGE of purified canine heart and liver ferritins. Samples are heart ferritin (2 μg, lane 1), liver ferritin (2 μg, lane 2), and marker proteins (2 μg each, lane 3): ovalbumin (45.0 kDa), lactate dehydrogenase (36.0 kDa), adenylate kinase (21.7 kDa), myoglobin (17.2 kDa), cytochrome *c* (12.4 kDa). H and L are ferritin subunits. Anode at bottom.

Figure 2 shows the difference in reactivity between canine heart and liver ferritins in a sandwich ELISA system developed for measurement of canine serum ferritin (Watanabe *et al.* 2000). The absorbance of heart ferritin was much lower than that of liver ferritin at any given concentration, showing that the former reacted much more weakly than the latter in the ELISA. As the H subunit was found to be predominant in canine serum ferritin in our previous study (Watanabe *et al.* 2000), H-subunit-rich heart ferritin was used as an ELISA standard for measuring canine serum ferritin in the following experiments.

Assay of ferritin in canine serum

Recoveries of 22.7 ng ml^{-1} and 45.5 ng ml^{-1} canine heart ferritin added to the canine sera diluted 11-fold with ELISA buffer were 96.6±9.2% (n=5) and 94.8± 6.8% (n=5), respectively. Intra-assay coefficients of variation from eight measures of ferritin in two canine sera were 2.4% (355 ± 8.6 ng ml^{-1}) and 9.1% (751 ± 68 ng ml^{-1}). Inter-assay coefficients of variation from six measures of ferritin in two canine sera were 10.8% (398 ± 43 ng ml^{-1}) and 4.8% (771 ± 37 ng ml^{-1}).

Serum ferritin from 51 apparently healthy dogs was determined. The ferritin concentration varied from 143 to 1766 ng ml^{-1}, with a mean value of 479 ± 286 ng ml^{-1}.

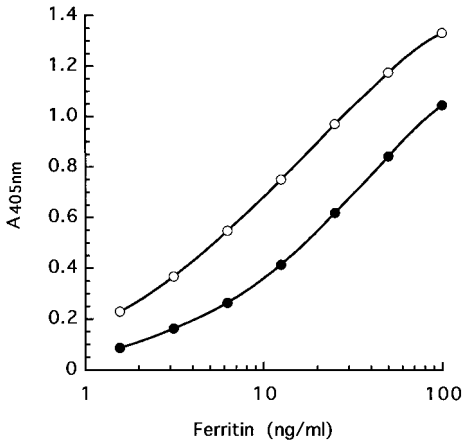

Figure 2. ELISA standard curves for canine heart and liver ferritins assayed with anti-rat liver ferritin antibodies. ●: canine heart ferritin, ○: canine liver ferritin.

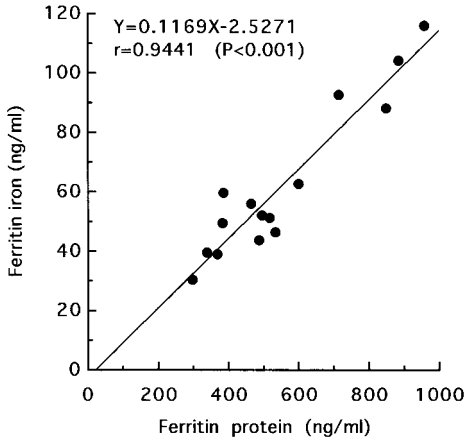

Figure 3. Relationship between serum ferritin protein and serum ferritin iron concentrations.

Iron content of canine serum ferritin

The mean overall recovery of iron from canine heart ferritin with an iron/protein ratio of 0.227 was 94.5 ± 12.1% (n=4), following its addition to canine serum to give a ferritin iron concentration of 68.6 ng ml^{-1}, heat-treatment at pH 4.8, and immunoprecipitation with rabbit antibodies to rat liver ferritin and goat antibodies to rabbit IgG.

In 15 dogs whose serum ferritin protein concentrations ranged from 298 to 959 ng ml^{-1}, serum ferritin iron concentrations varied from 30.4 to 115.9 ng ml^{-1}. Figure 3 shows the significant linear correlation between ferritin iron and protein concentrations (r=0.9441, P<0.001). The mean iron/protein ratio of serum ferritin was 0.112 ± 0.017.

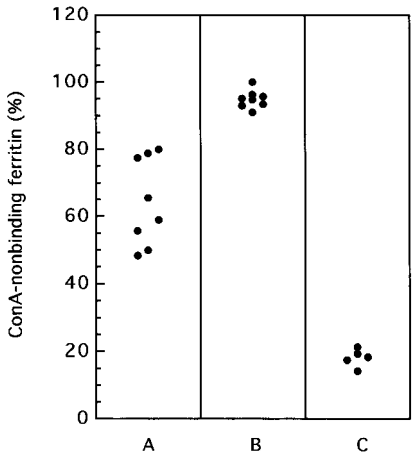

Figure 4. Binding of canine and human serum ferritin to ConA. Ferritin in untreated canine serum (A) and in the solutions which had been obtained by heat-treating canine (B) and human (C) sera at pH 4.8 was incubated with ConA-Sepharose 4B. Bound and free ferritins were separated by centrifugation. ConA-nonbinding ferritin was expressed as a percentage relative to free ferritin after incubation with Sepharose 4B.

Nonbinding of canine serum ferritin to ConA

Binding of ferritin in untreated canine serum was measured by incubating serum with ConA-Sepharose and separating bound and free ferritin by centrifugation. A significant proportion of the serum ferritin bound to the lectin (Figure 4A). However, little binding was found when ferritin in the solution obtained by heat-treating canine serum at pH 4.8 was incubated with ConA (Figure 4B). In contrast, a high proportion of ferritin derived from heat-treated human serum bound to the lectin (Figure 4C).

Discussion

The H and L subunits of ferritin are known to have a large immunological difference (Luzzago *et al.* 1986). H-subunit-rich canine heart ferritin was found in the present study to be much less reactive with antibody against rat liver ferritin, in which the L subunit was predominant, than L-subunit-rich canine liver ferritin. Our previous study revealed that the H subunit was predominant in canine serum ferritin (the mean H/L subunit ratio: 3.46 ± 1.12) (Watanabe *et al.* 2000). Therefore, if liver ferritin is used as an ELISA standard, the canine serum ferritin level will be underestimated. In fact, the concentration of ferritin in canine serum measured using a liver ferritin standard by Andrews *et al.* (1992) is about one third of that measured

using a heart ferritin standard in the present study. Because the H/L subunit ratio of canine heart ferritin is almost equal to that of canine serum ferritin, we believe that heart ferritin should be used as an ELISA standard for measuring serum ferritin.

A certain percentage of ferritin in canine serum precipitates by low-speed centrifugation at $16\,000 \times g$ due to autoantibody to ferritin (Watanabe *et al.* 2000). Therefore, if the serum were used directly for quantification of ferritin iron by immunoprecipitation, even a control immunoprecipitate obtained using normal rabbit IgG in place of rabbit antibody to rat liver ferritin would contain ferritin iron. In the present study, canine serum was heat-treated under an acid condition in order to remove ferritin-binding proteins, including the autoantibody to ferritin. After such a treatment, almost all ferritin was recovered in the supernatant obtained by low-speed centrifugation.

Canine serum ferritin was found to contain a significant amount of iron, its iron/protein ratio being about half that of purified canine heart ferritin. In contrast, human ferritin purified or immunoprecipitated from the serum or plasma of patients with iron overload had a relatively low iron/protein ratio of 0.02 to 0.07 (Worwood *et al.* 1976; Cragg *et al.* 1981; Pootrakul *et al.* 1988). Human serum ferritin contains only a trace of the H subunit (Cragg *et al.* 1981; Santambrogio *et al.* 1987) that has the ferroxidase activity (Levi *et al.* 1988, 1989), whereas canine serum ferritin is H-subunit-rich (Watanabe *et al.* 2000). The difference in iron content between human and canine serum ferritins may reflect the difference in their subunit compositions. Fetal bovine serum ferritin having a H/L subunit ratio of 0.03 to 0.27 has a relatively high iron content (a mean iron/protein ratio: 0.20) (Kakuta *et al.* 1997). These results suggest that there is a species-difference in the iron content of serum ferritin.

Our previous study showed that canine serum contained autoantibodies (IgM and IgA) reacting with ferritin (Watanabe *et al.* 2000). Apparent binding of canine serum ferritin to ConA was observed (Figure 4A) in the present study. Since serum ferritin binding to the lectin was absent after heat-treating the canine serum to remove the ferritin-binding antibodies (Figure 4B), we speculate that the ferritin in the untreated serum binds indirectly to the lectin via the carbohydrate-containing autoantibodies. Human serum ferritin, which contains the glycosylated subunit G (Cragg *et al.* 1981; Santambrogio *et al.* 1987), bound to ConA even after heat-treating the human serum (Figure 4C). These results suggest that canine serum ferritin contains no glycosylated subunit, unlike human serum ferritin.

Previous studies on the clearance of ferritin from human plasma have shown that ^{131}I-human spleen ferritin is cleared very rapidly (a half-life $T^{1/2} =$ 9 min) (Cragg *et al.* 1983), whereas the ConA-binding fraction of ^{131}I-human plasma ferritin is removed extremely slowly ($T^{1/2}$ approximately 50 h) (Worwood *et al.* 1982). These results suggest that glycosylation is responsible for the relatively slow clearance of human plasma ferritin. In dogs, it has been found that liver ferritin is removed very rapidly from the circulation ($T^{1/2} < 10$ min) (Pollock *et al.* 1978). Although the clearance of canine serum ferritin is unknown, we speculate that it is cleared very rapidly because of the absence of the G subunit. A mean plasma ferritin iron concentration in normal humans with plasma ferritin protein levels of 20 to 80 ng ml^{-1} is reported to be 5 ± 4 ng ml^{-1} (Pootrakul *et al.* 1988). Therefore, the serum (or plasma) ferritin iron level is much higher in normal dogs than in normal humans. There may be a possibility that serum iron is transported by ferritin as well as by transferrin in dogs, provided that serum ferritin is cleared as rapidly as tissue ferritin. However, the physiological role of serum ferritin as an iron-transporter in dogs remains to be established.

Acknowledgements

We are grateful to Drs. Nobuyuki Susa and Shunji Ueno, Laboratory of Veterinary Public Health, School of Veterinary Medicine and Animal Sciences, Kitasato University, for carrying out the atomic absorption analysis.

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