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Purification and characterization of canine serum ferritin-binding proteins

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Abstract

Ferritin-binding protein (FBP) is known to interact with circulating ferritins in mammals. Canine FBPs were purified from canine serum by affinity chromatography and were identified as IgM, IgG, and IgA by immunoblotting with alkaline phosphatase-labeled antibodies to canine IgM, IgG, and IgA heavy chains. Following further purification by application to a Sephacryl S-300 column, canine FBPs were separated into 81.3- and 27.7-kDa bands by sodium dodecyl sulfate-polyacryamide gel electrophoresis, and the 81.3-kDa band reacted with the anti-canine IgM heavy chain antibody. Purified canine FBP bound to canine liver ferritin, but not to canine albumin and transferrin. FBP showed greater binding to the expressed bovine ferritin H-chain homopolymer than to the expressed bovine ferritin L-chain homopolymer. The binding of FBP with canine liver ferritin was dose-dependently inhibited by anti-rat liver ferritin antibody, and the anti-ferritin antibody dissociated the bound FBP in a dose-dependent manner, even after binding FBP with liver ferritin. The canine ferritin H subunit peptide fragment with amino acid residues 148–155 (NH_2-GDHVTNLR-COOH) in its C-terminal region was recognized by FBP. These results indicate that canine serum FBPs are autoantibodies to ferritin (IgM, IgG, and IgA) and that anti-ferritin autoantibody (IgM) recognizes the C-terminal region of ferritin H subunit.

Introduction

Ferritin is an iron-binding protein found in the organs and cells of mammals (Theil 1987; Harrison & Arosio 1996). The 24-mer globular protein has a molecular mass of kDa and contains a core of ferric iron atoms (2000–3000 Fe/molecule) (Theil 1987; Harrison & Arosio 1996). Tissue ferritin is composed of H (heart) and L (liver) subunits, each with distinct immunological and physiological properties (Luzzago *et al.* 1986; Theil 1987; Levi *et al.* 1988, 1989; Harrison & Arosio 1996). The H chain utilizes ferroxidase to incorporate iron, while the L chain does not contain ferroxidase, but adjusts the microenvironment of core and promotes core growth inside the ferritin shell by accelerating iron nucleation (Levi *et al.* 1988, 1989, 1992; Harrison & Arosio 1996).

In normal human, equine, bovine, porcine, canine, and feline sera, ferritin is found in relatively low concentrations (< 1 μg/ml), and ferritin levels are positively correlated with body iron reserves (Addison *et al.* 1972; Walters *et al.* 1973; Smith *et al.* 1984a, b; Miyata & Furugouri 1987; Andrews *et al.* 1992, 1994). Serum ferritins of some of these species have been characterized biochemically with respect to concanavalin A binding, iron content, and subunit composition (Worwood *et al.* 1979; Santambrogio *et al.* 1987; Kakuta *et al.* 1997; Watanabe *et al.* 2000a, b).

A number of ferritin-binding protein (FBP) types present in mammalian circulation have been described: H-kininogen in human serum (Torti &

Torti 1998), alpha-2-macroglobulin in rabbit (Santambrogio & Massover 1989) and horse (Massover 1994) sera, fibrinogen in horse plasma (Orino *et al.* 1993), and anti-ferritin autoantibody in canine (Watanabe *et al.* 2000a) and bovine (Orino *et al.* 2004) sera. Although the physiological roles of these FBPs have not been clearly established, FBPs are considered to be involved in the clearance of circulating ferritins after forming complexes (Covell *et al.* 1984; Massover 1994; Watanabe *et al.* 2000a). Horse fibrinogen inhibits serum ferritin immunoassay by competition with anti-ferritin antibodies binding to the ferritin epitope (Orino *et al.* 1993). Human H-kininogen blocks bradykinin release by binding the light chain of the H-kininogen with ferritin (Partharathy *et al.* 2002). However, although the anti-ferritin autoantibodies are expected to form immune complexes with circulating ferritins, it remains to be clarified whether complex formation is due to antibody–antigen reactions.

Previous data indicates that canine FBPs are autoantibodies to ferritin (IgM and IgA) (Watanabe *et al.* 2000a). The aim of this study is to purify and characterize canine FBPs.

Materials and methods

Materials

Alkaline phosphatase (ALP)-labeled antibodies to canine IgM, IgG, and IgA heavy chains were purchased from Bethyl Laboratories (Montgomery, TX, USA). Immuno Plate Maxisorp F96 and Immunomobilizer Amino microtiter plates were from Nunc (Roskilde, Denmark). CNBr-activated Sepharose 4B and Sephacryl S-300 were from Pharmacia (Uppsala, Sweden). Canine serum albumin and transferrin and rabbit serum IgG were from Sigma (St. Louis, MO, USA). Bovine serum albumin was from Boeringer Manheim (Germany). Centriplus YM-50 was from Millipore Corporation (Bedford, MA, USA).

Blood

Canine blood collected from clinically healthy beagles were kept at Kitasato University. After coagulation, serum was obtained by centrifugation at 1650×*g* for 15 min and kept at −20 °C.

Ferritin preparation

Canine liver ferritin was purified from pieces of frozen canine liver based on the method of Watanabe *et al.* (2000a). Bovine H- and L-chain ferritin homopolymers were prepared by expressing bovine ferritin H and L subunit cDNAs using a baculovirus expression system, as previously described (Orino *et al.* 2002).

Antibody to ferritin

Rabbit anti-rat liver ferritin antibody was prepared and purified as previously described (Watanabe *et al.* 2000a).

Protein measurement

Protein concentrations were determined using bovine serum albumin as the protein standard according to the method of Lowry *et al.* (1951).

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

SDS-PAGE was carried out according to the method of Schägger & von Jagow (1987), using 4.5% stacking gel and 10% running gel. Ferritin subunit bands were stained with Coomassie Brilliant Blue R250 and a densitometry scan was conducted at 565 nm using a Flying Spot Scanner (Shimadzu CS9000, Shimadzu Corp., Kyoto, Japan).

Immunoblot analysis was performed using procedures previously described by Orino *et al.* (2004).

Purification of FBPs by affinity chromatography

Canine serum (7 ml) was applied to a CNBr-activated Sepharose 4B gel column (10 ml; 2×3.3 cm) coupled with canine liver ferritin (10 mg) as the ligand and was equilibrated with phosphate-buffered saline (PBS: 150 mM NaCl, 20 mM sodium phosphate, pH 7.2) at a flow rate of 12 ml h^{-1}. The column was washed with PBS until the absorbance of the effluent was below 0.01 at 280 nm. Protein fractions were eluted in 3-ml fractions with PBS containing 3 M KSCN (pH 7.2). The absorbance of each fraction was measured at 280 nm, and peak fractions were immediately dialyzed against PBS. Peak fractions

were pooled and concentrated by centrifugation using Centriplus YM-50 and then applied to a Sephacryl S-300 column (2×100 cm) equilibrated with PBS. To examine ferritin-binding activity, an aliquot (100 μl) of each 3-ml fraction was diluted 4-fold with ELISA buffer containing 0.5 M $(NH_4)_2SO_4$ (pH 7.2) and was added to the wells of canine ferritin coated-microtiter plate (1 μg/well). The plate was subjected to ferritin-binding assay using ALP-labeled anti-canine IgM heavy chain antibody, as described below.

Ferritin-binding assay

Aliquots (100 μl) of 10 μg ml^{-1} canine liver ferritin, expressed bovine ferritin H- and L-chain ferritin homopolymers, canine serum albumin and transferrin in PBS were added to the wells of an Maxisorp F96 microtiter plate and incubated at 4 °C overnight. The protein-coated plate was washed and masked with ELISA buffer (PBS containing 0.1% gelatin and 0.1% Tween20) as previously described (Watanabe *et al.* 2000a). After washing, 100 μl of 10 μg ml^{-1} purified FBP in ELISA buffer containing 0.5 M $(NH_4)_2SO_4$ (pH 7.2) was added to each well and the plate was incubated at 37 °C for 2 h. After washing, 100 μl of ALP-labeled anti-canine IgM heavy chain antibody appropriately diluted in ELISA buffer was added. After a final incubation for 2 h at 37 °C, plates were washed and the enzyme reaction was carried out as previously described (Orino *et al.* 1993).

To elucidate the ferritin binding site (epitope) of the canine FBP, three different peptide fragments (peptide 87–95, NH_2-KPDRDDWEN-COOH; peptide 126–132, NH_2-DPHLCDF-COOH; and peptide 148–155: NH_2-GDHVTNLR-COOH) were synthesized (Thermo Electron Corporation, Ulm, Germany) according to the sequence data of canine liver ferritin H subunit (GenBank accession number AB175610, peptide fragment are numbered according to amino acid numbers of the H subunit sequence) based on the previous report on antigenic sites on the ferritin molecules (Addison *et al.* 1984). Aliquots (100 μl) of 125 μg ml^{-1} synthesized peptides or 1.25 μg ml^{-1} canine liver ferritin in 100 mM Na_2CO_3 (pH 9.6) were added to the wells of an Immobilizer Amino plate. Ferritin-binding activity was detected as described above except for using 2 mM cystine in 100 mM Na_2CO_3 (pH 9.6) for masking the well of the plate.

Results

Characterization of canine serum FBP

Immunoblotting of affinity-purified canine serum FBPs with ALP-labeled antibodies specific for canine IgM, IgG, and IgA heavy chains was carried out (Figure 1). Fractions eluted from the Sepharose 4B column without ligand did not produce any detectable bands when probed with these antibodies. Immunoblotting of affinity-purified fractions obtained from affinity chromatography with ligand showed bands corresponding to IgM, IgG, and IgA heavy chains. However, two of three bands detected with anti-canine IgM heavy chain antibody corresponded to the bands detected with anti-canine IgG and IgA heavy chain antibodies, and one of two bands detected by anti-canine IgG heavy chain antibody corresponded to a band strongly detected by the anti-IgM antibody. Detection of bands by different classes of antibodies seemed to be due to immunological cross-reactions between heavy chains or contamination of different heavy chains in the antigens used to prepare the test antibodies. FBP purified

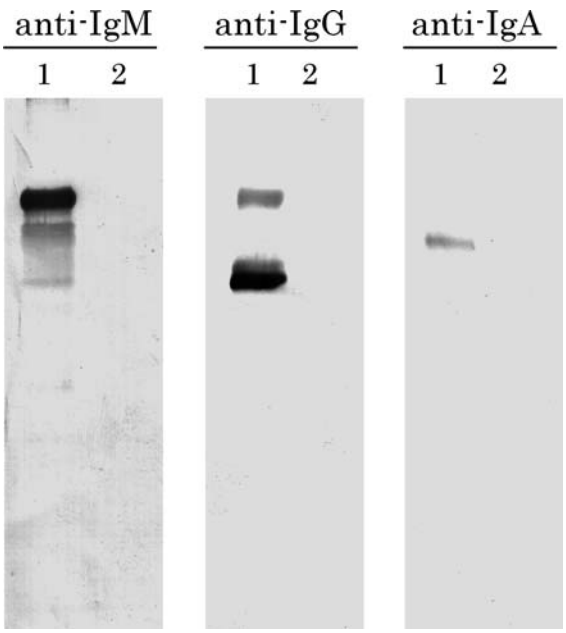

Figure 1. Immunoblotting analysis of affinity-purified canine serum FBPs. Fractions eluted from canine liver ferritin–Sepharose 4B column (lane 1) and from Sepharose 4B without ligand (lane 2) were applied to SDS-PAGE followed by immunoblotting with ALP-labeled antibodies to canine IgM, IgG, and IgA heavy chains.

from canine serum by sequential affinity and Sephacryl S-300 columns separated into 81.3-kDa and 27.7-kDa bands on SDS-PAGE (Figure 2a), and the 81.3-kDa band was detected more strongly by anti-canine IgM heavy chain than by anti-canine IgG heavy chain antibody (Figure 2b), suggesting that anti-canine IgG heavy chain antibody cross-reacted with the IgM heavy chain as previously described. Finally, purified FBP was identified as IgM, which binds to canine ferritin, and was used in the following experiments.

Specificity of canine FBP to ferritin

Purified canine FBP specifically bound to ferritin, but not to canine albumin and transferrin, as shown in Figure 3. To clarify the specificity of FBP to ferritin subunits, bovine H- and L-chain ferritin homopolymers expressed in a baculovirus expression system were also tested. FBP showed greater binding to the expressed bovine H-chain homopolymer than to the expressed bovine L-chain homopolymer, and canine liver ferritin with subunit ratio of H/L = 4.3 showed binding activity intermediate between the H and L chain homopolymers.

The binding of canine FBP with canine liver ferritin was inhibited by rabbit anti-rat liver ferritin antibody in a dose-dependent manner (Figure 4a). Even after binding of canine FBP to the ferritin-coated plate, the anti-rat liver ferritin antibodies removed the bound FBP from the well (Figure 4b). Rabbit IgG did not affect binding of FBP with canine ferritin.

For the identification of ferritin epitope of canine IgM, three different peptide fragments were used as described in the Materials and methods. Figure 5 shows that canine FBP reacted only with peptide 148–155, but not with peptides 87–95 and 126–132.

Discussion

Canine serum FBPs have been identified as autoantibodies to ferritin (IgM and IgA) (Watanabe *et al*. 2000a). In this study, canine IgG was also identified as an FBP by immunoblotting analysis of affinity-purified canine FBPs. These FBPs were shown to specifically bind to canine ferritin as the FBPs did not absorb on the Sepharose 4B column in the absence of ligand. Canine IgG was detected following the concentration of FBPs by affinity

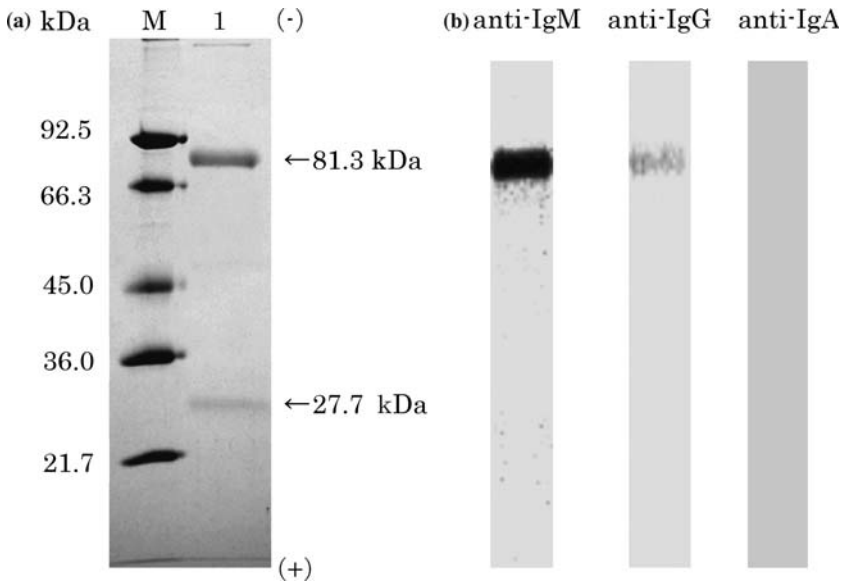

Figure 2. SDS-PAGE (a) and immunoblotting (b) analyses of purified FBPs by sequential chromatography on affinity and Sephacryl columns. (a) Purified canine FBPs (2 μg, lane 1) were run in a 10% polyacrylamide gel against the marker proteins (lane M, 1 μg each: phospholylase b, 92.5 kDa; serum albumin, 66.3 kDa; ovalbumin, 45.0 kDa; lactate dehydrogenase, 36.0 kDa; and adenylate kinase, 21.7 kDa. (b) Canine FBPs (1 μg) were applied to SDS-PAGE and then subjected to immunoblotting with ALP-labeled antibodies to canine IgM, IgG, and IgA heavy chains.

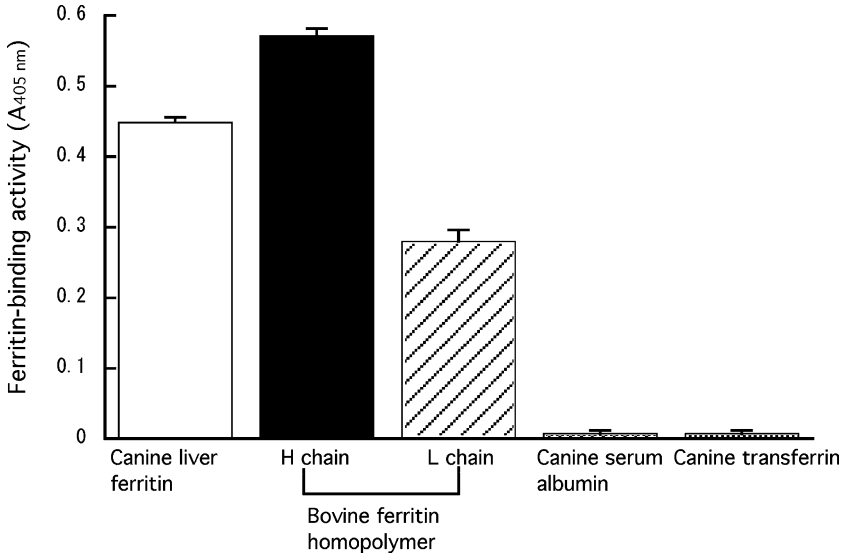

Figure 3. Specific binding of purified FBP to ferritin. A 100-μl aliquot of FBP (10 μg ml^{-1}) in ELISA buffer containing 0.5 M $(NH_4)_2SO_4$ was applied to a protein-coated plate (1 μg/well) and detected with ALP-labeled anti-canine IgM heavy chain antibody. Wells were coated with the following proteins: canine liver ferritin (H/L = 0.43) (open bar); expressed bovine ferritin H-chain (solid bar) and L-chain (hatched bar) homopolymers; canine serum albumin (cross-hatched bar); canine transferrin (gray bar). Each bar represents the mean ± SD of four determinations.

chromatography and could be detected by the more sensitive immunoblotting but not by plate assay with ALP-labeled anti-canine IgG heavy chain antibody.

In canine, anti-ferritin autoantibodies may be involved in rapid sequestration of circulating ferritin after immune complex formation with ferritin through reticuloendothelial cells (Watanabe *et al.* 2000a). Canine serum ferritin is considered to be an iron transporter based on results of rapid clearance tests of biotinylated canine ferritin ($T_{1/2}$ = 18.0 min) (Orino *et al.* 2003). Canines used in tests did not show any clinical signs of autoimmune disease despite the presence of FBPs. The complexation of ferritin with anti-ferritin autoantibodies in canine circulation did not seem to cause clinical disease.

In this study, canine IgM was purified as an anti-ferritin autoantibody by additional Sephacryl S-300 gel filtration. Canine IgM specifically bound to canine liver ferritin, and bound more to the expressed bovine ferritin H-chain homopolymer than to expressed bovine ferritin L-chain homopolymer, supporting previous findings that canine serum anti-ferritin autoantibodies more readily recognize the H subunit than the L subunit (Orino *et al.* 2002). Amino acid sequences of canine H and L subunits show high homology with bovine H and L subunits, respectively (H: 92%; L: 92%) (Orino *et al.* in press). Immunological properties of ferritin depend on the subunit composition, due to large immunological differences between H and L subunits (Luzzago *et al.* 1986). This also explains why canine liver ferritin with subunit ratio of H/L = 4.3 showed binding activity intermediate between the H- and L-chain homopolymers.

Although canine anti-ferritin autoantibody binds to serum and tissue ferritins (Watanabe *et al.* 2000a), whether these antibodies form complexes through antigen–antibody reaction remains to be clarified. Use of sandwich ELISA for the measurement of canine serum ferritin was not compromised by interference due to the competitive inhibition of anti-ferritin antibody with ferritin epitope by canine FBPs (Watanabe *et al.* 2000a). Ferritin-binding activity was not detected in serum protein- or FBP-coated microtiter plates incubated with canine liver ferritin followed by detection with the same ALP-labeled rabbit anti-rat liver ferritin antibodies that were used in the ferritin immunoassay (data not shown). The present study showed that the rabbit-anti rat ferritin antibodies in sandwich ELISA inhibited the binding of canine FBP to canine liver ferritin and dissociated the bound FBP, even after binding FBP with canine liver ferritin. Since canine FBPs

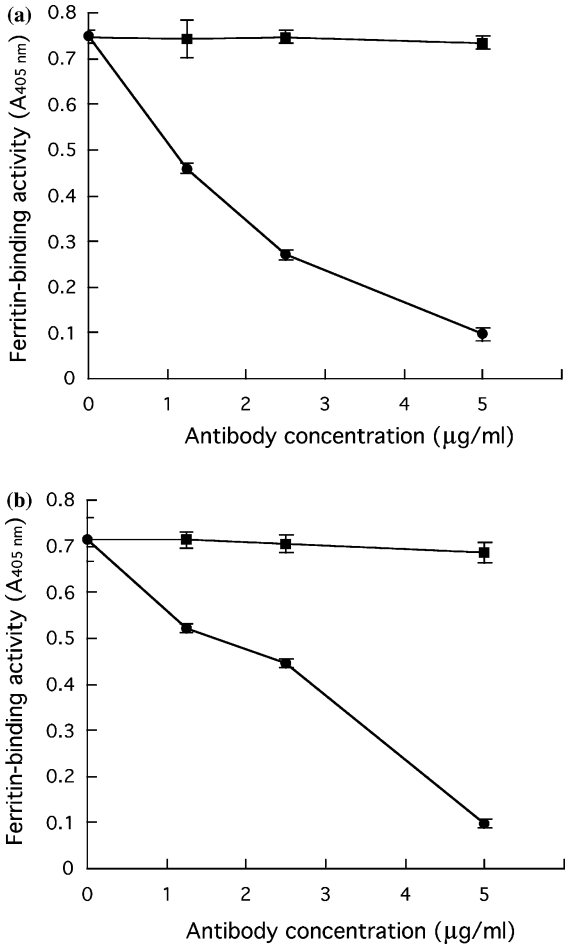

Figure 4. Inhibitory effects of anti-rat liver ferritin antibody on the binding of purified FBP to canine liver ferritin. (a) A 100-μl aliquot of the FBP (10 μg ml^{-1}) in ELISA buffer containing 0.5 M $(NH_4)_2SO_4$ were added to each well of canine liver ferritin-coated plate (1 μg/well) along with various amounts of purified anti-rat liver ferritin antibody (solid circles) and detected with ALP-labeled anti-canine IgM heavy chain antibody. Rabbit IgG (solid squares) was used as a control in the place of anti-ferritin antibody. Each bar represents the mean ± SD of four determinations. (b) After binding FBP to the ferritin-coated plate as in (a) in the absence of anti-ferritin antibody, plate was incubated with ALP-labeled anti-canine IgM heavy chain antibody in the presence of the anti-rat liver ferritin antibody or the rabbit IgG.

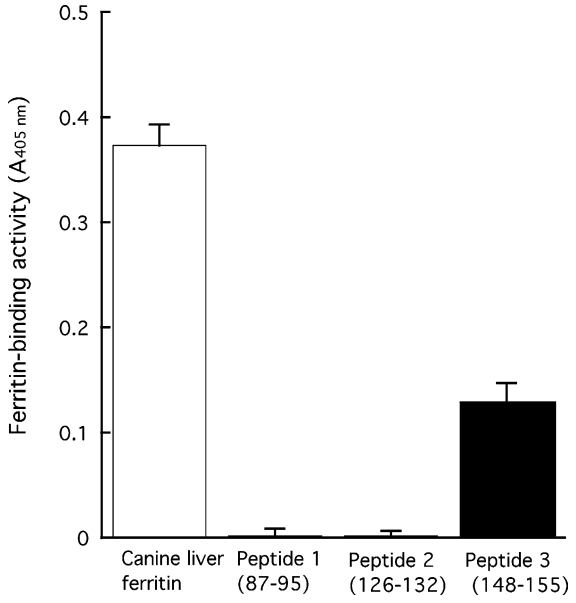

Figure 5. Binding assay of purified FBP to the synthesized peptides. A 100-μl aliquot of peptide fragment (125 μg ml^{-1}) or canine liver ferritin (1.25 μg ml^{-1}) in 100 mM Na_2CO_3 (pH 9.6) was coated on each well of an Immobilizer Amino microtiter plate. Aliquots (100 μl) of the purified FBP (10 μg ml^{-1}) in ELISA buffer containing 0.5 M $(NH_4)_2SO_4$ were added to each well of peptide- or ferritin-coated plate followed by detection with ALP-labeled anti-canine IgM heavy chain antibody. Bars indicate canine liver ferritin (open bar), peptide 87–95 (hatched bar), peptide 126–132 (cross-hatched bar), and peptide 148–155 (solid bar). Peptide fragments were numbered according to amino acid number of the canine liver ferritin H subunit sequence (GenBank accession No. AB175610). Each bar represents the mean ± SD of four determinations.

have lower ferritin-binding activity to canine ferritin than the anti-ferritin antibodies, canine FBPs did not affect sandwich ELISA and their ferritin-binding activities were not detected by the anti-rat liver ferritin antibody. These results also provide indirect evidence of specific binding of canine FBP with ferritin through antigen–antibody reactions.

Horse ferritin L subunit consists of a bundle of four long helices, A (residues 10–39), B (45–72), C (92–120), D (124–155), with a fifth helix, E (160–169) and a long loop (73–91) connecting helices B and C (Ford *et al.* 1984), and mammalian ferritin H and L subunits have similar overall structures including canine H and L subunits (Ford *et al.* 1984; Theil 1987; Harrion & Arosio 1996; Orino *et al.* 2005). Antigenic sites of ferritin molecules are located in continuous inter-helical regions of the polypeptide chains on the molecular surface and encompass amino acids remote in the primary structure or belonging to different subunit (Addison *et al.* 1984). We used three different peptide fragments of peptide 87–95, 126–132, and 148–155 which are located on the surface of ferritin molecule in the loop, the CD turn, and the the DE turn, respectively. Purified FBP reacted only with peptide 148–155 in the C-terminal region of ferritin H subunit, but not with peptides 87–95 and 126–132. The peptide 148–155 fragment is expected to be

located on the ferritin surface near the non-helical region of DE turn in the C-terminal of ferritin H subunit, although other surface regions in the synthesized fragments (peptides 87–95 and 126–132) did not show any reaction. In the canine L subunit, there is a one-amino acid substitution from valine to leucine in this region (Orino *et al.* 2005). Because FBP binds with the bovine L chain homopolymer, this fragment region may be common to both H and L subunits in canine FBP. However, anti-rat ferritin antibody did not bind with any fragment synthesized in this study (data not shown). Although the specific epitope of anti-ferritin antibody was not identified, the antibody may recognize an epitope or conformational region near a specific binding region of canine FBP (IgM). To our knowledge, this report is the first to identify an antigenic determinant on the ferritin molecule for anti-ferritin autoantibody. This study will help to characterize of the binding of anti-ferritin autoantibody with circulating ferritins.

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