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Characterization of feline serum ferritin-binding proteins: the presence of a novel ferritin-binding protein as an inhibitory factor in feline ferritin immunoassay

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Abstract Ferritin-binding proteins (FBPs) such as anti-ferritin antibody, α-2-macroglobulin, apolipoprotein B are expected to interact with circulating ferritin to eliminate it from circulation. However, we found that feline serum more strongly inhibits the detection of canine liver ferritin by immunoassay than its apoferritin; putative FBPs probably conceal ferritin epitopes detected by anti-ferritin antibodies. After complex formation between affinity-purified FBPs and canine liver ferritin, co-immunoprecipitates of the complex by anti-bovine spleen ferritin antibody were found to contain autoantibodies (IgG, IgM, and IgA) to ferritin by immunoblot analysis with antibodies specific for feline IgG, IgM, and IgA. On the other hand, affinity-purified samples did not show any inhibitory effect in the ferritin immunoassay. This result shows that feline serum has another FBP, which inhibits ferritin immunoassays, but not anti-ferritin autoantibody. A feline FBP was partially purified from feline serum by $(NH_4)_2SO_4$ fractionation (33–50%), gel filtration chromatography, and anion exchange chromatography. After binding of the partially purified sample with canine liver ferritin coupled-Sepharose gel, the FBP was separated and purified from complexes formed in a native-PAGE gel. SDS–PAGE analysis showed that the purified FBP is a homomultimer composed of 31 kDa monomeric subunits connected by intermolecular disulfide bonds. Detection of feline liver ferritin by immunoassay was inhibited by FBP in a dose-dependent manner. The purified protein molecules appeared to be conglomerate of pentraxin-like molecules by its electron micrographic appearance. These results demonstrate that feline serum contains a novel FBP as inhibitory factor of ferritin immunoassay with different molecular properties from those of other mammalian FBPs, in addition to auto-antibodies (IgG, IgM, and IgA) to ferritin.

Keywords Anti-ferritin autoantibody · Ferritin · Ferritin-binding protein · Immunoassay

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Introduction

Ferritin, an iron-binding protein, is an ubiquitous protein in all living organisms, from microorganisms to mammals (Theil 1987; Andrews et al. 1992). It is a 24-mer composed of heart (H) and liver (L) subunits,

with molecular masses of 21 and 19 kDa, respectively; the H and L subunit ratio depends on the cell type and tissue (Theil 1987; Andrews et al. 1992). In mammals, ferritin circulates in the blood in relatively low concentrations (1 μg ml^{-1}) as well as in tissues (Addison et al. 1972; Andrews et al. 1994; Orino and Watanabe 2008).

A variety of ferritin-binding proteins (FBPs) in mammalian serum and/or plasma have been described: H-kininogen (Torti and Torti 1998) and apolipoprotein B (apoB) (Seki et al. 2008) in human serum; α-2-macroglobulin in rabbit and horse serum (Santambrogio and Massover 1989; Massover 1994); anti-ferritin autoantibody in canine, horse, and bovine serum (Orino et al. 2004, 2006a,b); and fibrinogen in horse plasma (Orino et al. 1993b). Although the physiological roles of these FBPs have not been fully established, FBPs may be involved in the clearance of circulating ferritins after forming complexes with them (Covell et al. 1984; Santambrogio and Massover 1989; Orino et al. 2004, 2006a,b). In mammals, although α-2-macroglobulin is known to be a common FBP (Santambrogio and Massover 1989; Massover 1994), we proposed that mammalian anti-ferritin autoantibody is also a common FBP (Orino and Watanabe 2008).

There are two mechanisms of binding between FBP and ferritin: direct binding with FBP (Parthasarathy et al. 2002; Orino et al. 1993b) and indirect binding with heme on the surface of ferritin (heme-binding protein) (Seki et al. 2008). However, FBPs inhibit ferritin immunoassays by concealing the ferritin epitope that is directed to anti-ferritin antibody (Niitsu et al. 1988; Orino et al. 1993a).

Although Andrews et al. (1994) developed a feline serum ferritin immunoassay; they did not find inhibitory effects of feline serum on the assay. However, we found strong inhibition of ferritin detection by feline serum. In this study, we demonstrate that feline serum contains anti-ferritin autoantibody and a novel FBP, which inhibits the ferritin immunoassay.

Materials and methods

Chemicals

Alkaline phosphatase (ALP)-conjugated NeutrAvidin, EZ-Link sulfo-NHS-biotin, and Coomassie Plus-The Better Bradford Assay kit were from Pierce Chemical Co. (Rockford, IL, USA). Goat anti-feline IgM, IgG (Fc), and IgA were from Betyl Laboratories, Inc. (Montgomery, TX, USA). ALP labeled rabbit anti-goat IgG (Fc) antibody was from American Qualex International, Inc. (San Clemente, CA, USA). Immuno Plate Maxisorp F96 and assay microplates for protein assay were from Nunc (Roskilde, Denmark) and Iwaki brand, Scitech Div. (Funabashi, Chiba, Japan), respectively. Bovine serum albumin was from Roche Diagnostics (Mannleria, Germany). Midi GeBAflex-tube was from Gene Bio-Application, Ltd (Kfar-Hanagid, Israel). CNBr-activated Sepharose 4B and Sephacryl S-300 were from Pharmacia (Uppsala, Sweden). DE-52 cellulose was from Whatman Biochemical (Clifton, NJ, USA). Vivascience Vivaspin centrifugal concentrator (100,000 molecular weigh cut off: MWCO) was from Vivascience-Sartorius (Hannover, Germany).

Ferritin and apoferritin preparation

Feline, canine, and rat liver ferritin monomers were purified from pieces of frozen feline, canine, and rat livers, respectively, as described previously (Kakuta et al. 1997; Orino et al. 2004). Canine liver apoferritin was prepared by dialysis with 100 mM thioglycollic acid in 100 mM acetate buffer (pH 5.5) followed by phosphate-buffered saline (PBS, 150 mM NaCl, 20 mM sodium phosphate, pH 7.2).

Antibodies

Purified antibodies specific to rat liver and bovine spleen ferritins were obtained by affinity chromatography of rabbit antisera obtained by immunization of rabbits with respective ferritins (Watanabe et al. 1998; Orino et al. 2006b). Biotin labeling of antibodies to ferritin was performed using EZ-Link sulfo-NHS-biotin according to the manufacturer's instruction.

Protein determination

Protein concentration was determined by Coomassie Plus-The Better Bradford Assay kit using bovine serum albumin as the standard according to the microplate protocol.

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

SDS–PAGE was carried out according to the method of Laemmli (1970) and Schägger and von Jagow (1987) using a 4.5% stacking gel and 10% running gel. Native-PAGE was performed using a 5% slab polyacrylamide gel and the buffer system of Davis (1964). Protein bands separated by each PAGE 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). Briefly, protein samples separated on an SDS–PAGE were exposed to goat anti-cat IgM, IgG (Fc), or IgA followed by ALP-labeled polyclonal antibodies specific for goat IgG (Fc). ALP-labeled antibodies bound on the membrane were detected with 100 mM Tris/Hcl (pH 7.6) containing 5 mM $MgCl_2$, 0.39 mM nitro blue tetrazolium, and 0.38 mM 5-bromo-4-chloro-3-indolylphosphate.

Sandwich ELISA

The determination of ferritin was performed by sandwich ELISA according to the procedure described previously (Watanabe et al. 1998). The concentrations of the affinity-purified anti-rat liver ferritin antibody and the biotinylated anti-rat liver ferritin antibody were 400 ng ml^{-1} and 250 ng ml^{-1}, respectively. ALP-conjugated NeutrAvidin at a concentration of 1 μg ml^{-1} was used for detection of the biotinylated anti-ferritin antibody. To examine the inhibitory effect on the ferritin immunoassay by FBP, known amounts of ferritin samples were added to the serum, eluted fractions, or FBP samples, and the mixture was subjected to sandwich ELISA.

Partial purification of anti-ferritin autoantibody by affinity chromatography

Feline 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 PBS 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 0.5 M $(NH_4)_2SO_4$ followed by 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.

Purification of a novel feline FBP

Feline serum (10 ml) was diluted with the same amount of PBS and brought to 33% saturation with solid $(NH_4)_2SO_4$, and then precipitates were removed by centrifugation at 13,000×*g* for 15 min. The supernatant obtained was brought to 50% saturation with solid $(NH_4)_2SO_4$ to give a precipitate which was removed by centrifugation at 13,000×*g* for 15 min. The latter precipitate was dissolved in PBS, and dialyzed three times against 1 l PBS followed by centrifugation at 13,000×*g* for 15 min to remove insoluble materials. The supernatant was concentrated with a Vivasciecne Vivaspin 6 centrifugal concentrator (100,000 MWCO) by centrifugation at 3,000×*g*, and the concentrated sample was applied to a Sephacryl S-300 column (2 × 100 cm) equilibrated with PBS at a flow rate of 12 ml h^{-1}. To examine the inhibition of ferritin detection, an aliquot (100 μl) of each 3 ml fraction was diluted 100-fold with ELISA buffer containing 0.5 M $(NH_4)_2SO_4$ (pH 7.2) and feline liver ferritin (5 ng ml^{-1}), and the mixture was subjected to sandwich ELISA. The fractions which inhibited ferritin detection were pooled, and were dialyzed against 1 l of 125 mM Tris/Hcl (pH 7.5). The dialyzed solution was applied to a DE-52 column (1 × 5 cm) equilibrated with 125 mM Tris/Hcl (pH 7.5) and washed with the same buffer. The column was washed with PBS until the absorbance of the effluent was below 0.01 at 280 nm. The protein was eluted from the column in 3-ml fractions with 200 mM Tris/Hcl (pH 7.5), and the absorbance of each fraction was measured at 280 nm, and peak fractions were immediately pooled. The eluted protein samples (15 μg) were mixed with PBS (1 ml) containing 5 μl canine liver ferritin coupled-Sepharose 4B gel (1 mg gel ml^{-1}), and incubated for 30 min at 37°C. After incubation, the gel was precipitated by centrifugation at 3,000×*g* for 10 min. The supernatant obtained was discarded and the precipitated gel was washed three times with 1 ml of PBS under the

same centrifugation conditions. After washing, the gel was suspended with sample buffer and applied to native PAGE. Protein bands were stained with 0.1% Coomassie Brilliant Blue R-250. In the purification step, gel slices corresponding to the stained band were excised from the gel and the desired protein was eluted by electro-elution using Midi GeBAflex-tube according to manufacturer's instructions.

Electron microscopy

Proteins were examined by the negative staining according to the method described by Morimatsu et al. (1989).

Results

Inhibitory effect of feline serum on the detection of canine ferritin in the immunoassay

The SDS–PAGE pattern of purified feline and canine liver ferritins used in this study was shown in Fig. 1A. The molecular masses of H and L subunits of feline and canine liver ferritins were 21.1 kDa and 19.3 kDa, respectively. The H/L subunit ratios of feline and canine liver ferritins were 2.5 and 2.4, respectively. Standard curves of feline and canine liver ferritins in the immunoassay developed in this study showed almost the same dose response (data not shown). Various amounts of feline serum were added to canine liver ferritin or its apoferritin to a final concentration of 5 ng ml^{-1}, and the mixture was subjected to sandwich ELISA. Feline serum strongly inhibited the detection of canine liver ferritin and its apoferritin in the immunoassay (Fig. 1B). Detection of canine liver ferritin by feline serum was significantly inhibited as compared with its apoferritin.

Characterization of affinity-purified FBP

Partially purified feline FBPs were obtained from feline serum by affinity chromatography. Proteins bound to the affinity column were eluted with PBS containing 0.5 M $(NH_4)_2SO_4$ (pH 7.2) followed by elution with PBS containing 3 M KSCN (pH 7.2). To characterize affinity-purified samples, after complex formation of the former

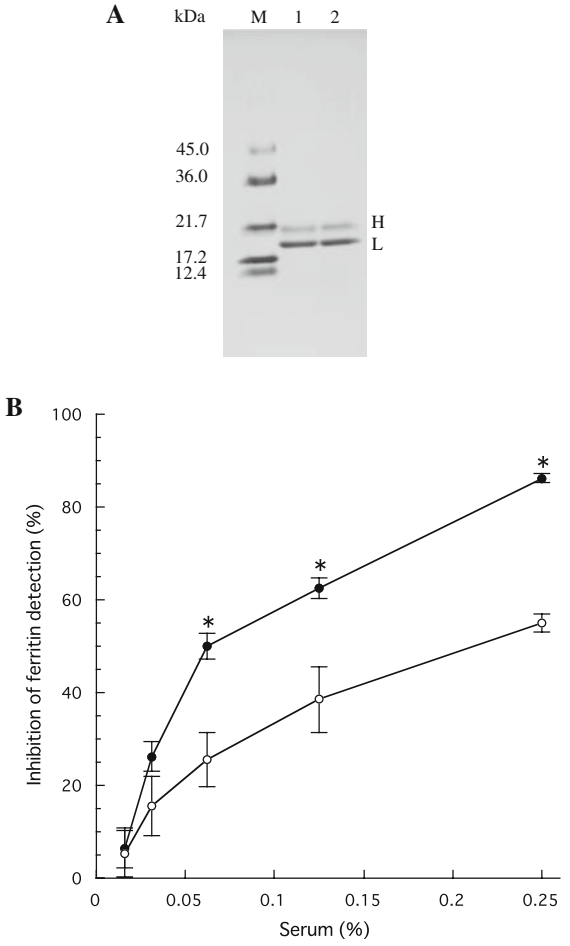

Fig. 1 Inhibition effect of feline serum on the detection of canine liver ferritin and its apoferritin by sandwich ELISA. **A** SDS–PAGE of purified feline and canine liver ferritins used in this study. Feline and canine liver ferritins (2 μg each) were applied to 10% SDS–PAGE using the buffer system of Schägger and von Jagow (1987) in reducing conditions (*lane 1*: feline ferritin; *lane 2*: canine ferritin). On the left are molecular mass marker proteins (M, 2 μg each). H and L indicate H and L subunits, respectively, of feline and canine liver ferritins. **B** Known amounts of canine liver ferritin and its apoferritin were added to a final concentration of 5 ng ml^{-1} to various amounts of ferine serum diluted with ELISA buffer containing 0.5 M $(NH_4)_2SO_4$ (pH 7.2), and the mixture was subjected to sandwich ELISA to detect ferritin. The value obtained in the absence of feline serum was defined as no inhibition. Each value is the mean ± SD of four replicates. The $*P < 0.01$, compared with the inhibition of detection of canine liver apoferritin

0.5 M $(NH_4)_2SO_4$ eluate (10 μg) and canine liver ferritin (10 μg), co-immunoprecitation of the complex by rabbit anti-bovine spleen ferritin antibody

(10 μg) was performed, and co-immunoprecipitates were subjected to immunoblot analysis with polyclonal antibodies specific for each H chain of feline IgG, IgM, and IgA (Fig. 2). Although feline immunoglobulins (IgG and IgM) were precipitated by the same centrifugation from the affinity-purified sample, the precipitated amount was negligible. Rabbit anti-bovine spleen ferritin antibody formed an immunocomplex with canine liver ferritin, and each antibody specific for the H chains of feline IgG, IgM, and IgA was found to cross-react with the H chain of rabbit IgG. As co-immunoprecitated complexes formed between affinity-purified FBPs and canine liver ferritin by rabbit anti-bovine spleen ferritin antibody, each H chain of feline IgG, IgM, and IgA was detected as a band migrating more slowly than the H chain of rabbit IgG, although the H chain of feline IgG was only slightly slower. The latter 3 M KSCN eluate was also found to contain anti-ferritin autoantibodies (IgG, IgM, and IgA) (data not shown). However, although affinity-purified samples eluted with 0.5 M $(NH_4)_2SO_4$ were added to known amounts of feline liver ferritin, there was no inhibitory effect on the detection of ferritin observed by immunoassay (Table 1).

Table 1 The effect of affinity-purified feline anti-ferritin autoantibodies on the detection of feline liver ferritin[a]

Affinity-purified sample (μg ml^{-1})	Detected ferritin (ng ml^{-1})	Detected (%)
0.31	3.4	100
0.63	3.3	97
1.25	3.3	97
2.50	3.3	97
5.00	3.3	97

[a]Known amounts of feline liver ferritin were added to a final concentration of 3.4 ng ml^{-1} to various amounts of affinity-purified fraction eluted with 0.5 M $(NH_4)_2SO_4$. The mixture was then subjected to sandwich ELISA to measure the ferritin level

Purification of a novel feline FBP

A novel feline FBP was partially purified from feline sera by $(NH_4)_2SO_4$ fractionation (33–50%), Sephacryl S-300 gel filtration, and DE-52 anion-exchange chromatography. The partially purified sample showed multiple bands including a major 31 kDa band on SDS–PAGE (data not shown). After binding of partially purified samples with canine liver ferritin coupled-Sepharose 4B, further purification was carried out by electro-elution from native PAGE, which

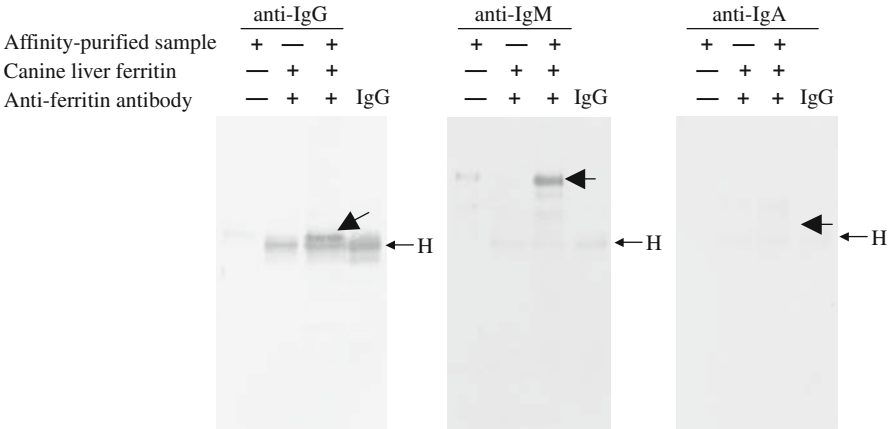

Fig. 2 Co-immunoprecipitation of complexes formed between partially purified feline FBP and canine liver ferritin by rabbit anti-bovine spleen ferritin antibody. Affinity-purified protein fractions (10 μg) eluted with 0.5 M $(NH_4)_2SO_4$ were incubated with canine liver ferritin (10 μg) at 4°C overnight, and then rabbit anti-bovine spleen ferritin antibody (10 μg) was added to the mixture. The mixture was centrifuged at 1,700×*g* for 10 min, and the resulting pellet was washed three times with 1 ml of PBS. After washing, the resulting pellet was separated on a 10% SDS-gel with the buffer system of Laemmili (1970), and subjected to immunoblotting with goat antibody specific for the H chains of feline IgM, IgG, or IgA, followed by detection of respective bound antibody by ALP-labeled rabbit antibody to goat IgG(Fc). Rabbit IgG (IgG) was run to discriminate the H chain of feline immunoglobulins with each sample (200 ng/lane). Larger and smaller arrows indicate the H chains of each feline immunoglobulin (IgG, IgM, or IgA) and the H chain(H) of rabbit IgG, respectively

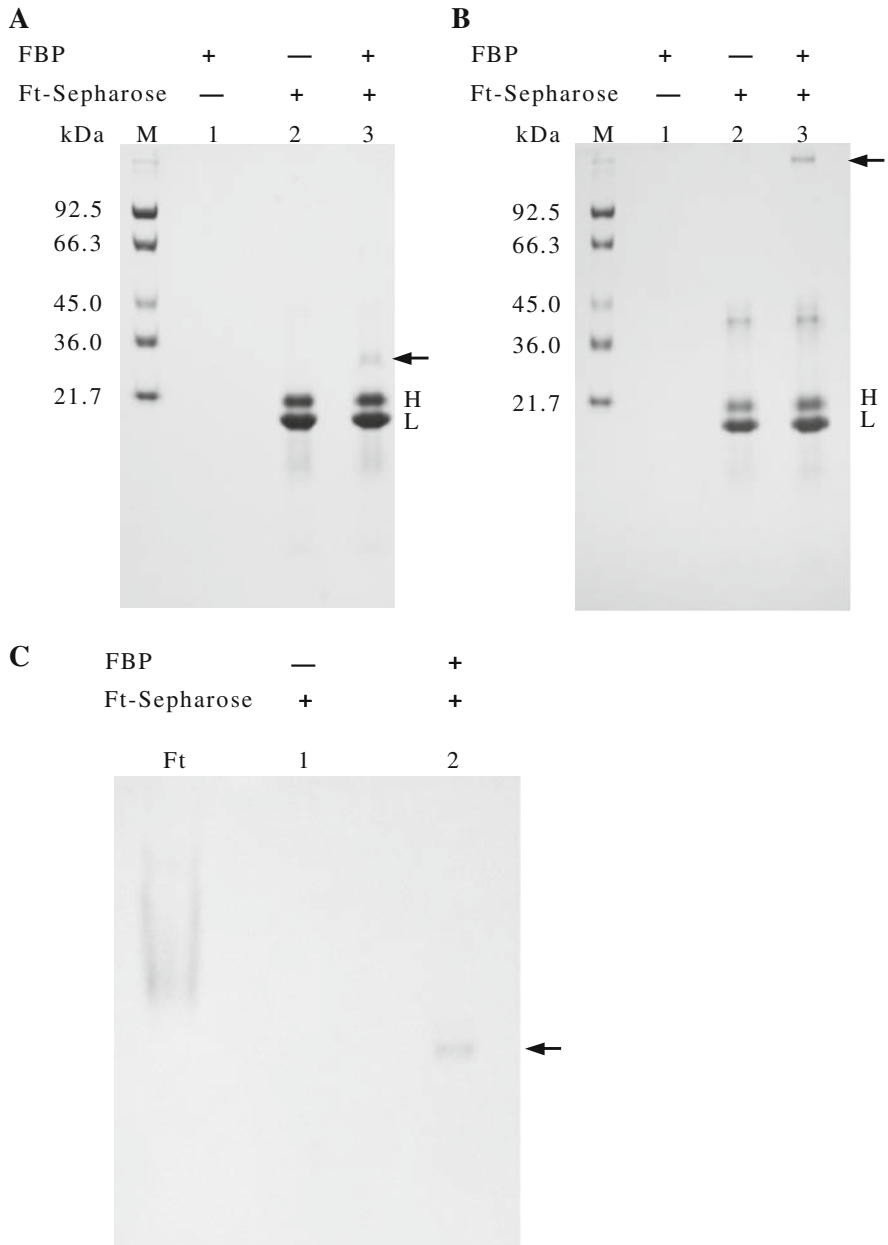

Fig. 3 The binding of a novel feline FBP with canine liver ferritin-coupled Sepharose 4B. Partially purified novel FBP (15 μg) was mixed with PBS (1 ml) containing 5 μl canine liver ferritin coupled-Sepharose 4B gel (1 mg gel ml^{-1}), and incubated for 30 min at 37°C. After incubation, the gel was precipitated by centrifugation at 3,000×*g* for 10 min, and the resulting pellet (gel) was applied to 10% SDS–PAGE using the buffer system of Schägger and von Jagow (1987) in the presence (**A**) or absence (**B**) of 40 mM DTT or Native-PAGE (**C**). **A** and **B** On the left are molecular mass marker proteins (M, 2 μg each). H and L indicate H and L subunits of canine ferritin. **C** Canine liver ferritin (4 μg/lane, Ft) was run with each sample. Arrow indicates the novel FBP

separated the FBP from the complex. The binding of the FBP to Ft-Sepharose was revealed by separation on SDS–PAGE (Fig. 3A, B). Partially purified FBP was not precipitated by centrifugation without incubation with canine liver ferritin coupled Sepharose 4B. Only Ft-Sepharose gel precipitate was able to separate H and L subunits of ferritin on SDS gels under reducing conditions, and dimers of ferritin subunits were detected as 39 kDa bands in addition to H and L subunit bands under non-reducing conditions. In SDS–PAGE of complexes formed between novel FBP and Ft-Sepharose, the novel FBP showed 30 kDa and 130 kDa bands in reducing and non-reducing conditions, respectively. Native PAGE of complexes formed between novel FBP and Ft-Sepharose showed only one band which migrated faster than the canine ferritin monomer, although native PAGE of Ft-Sepharose 4B only (no FBP)

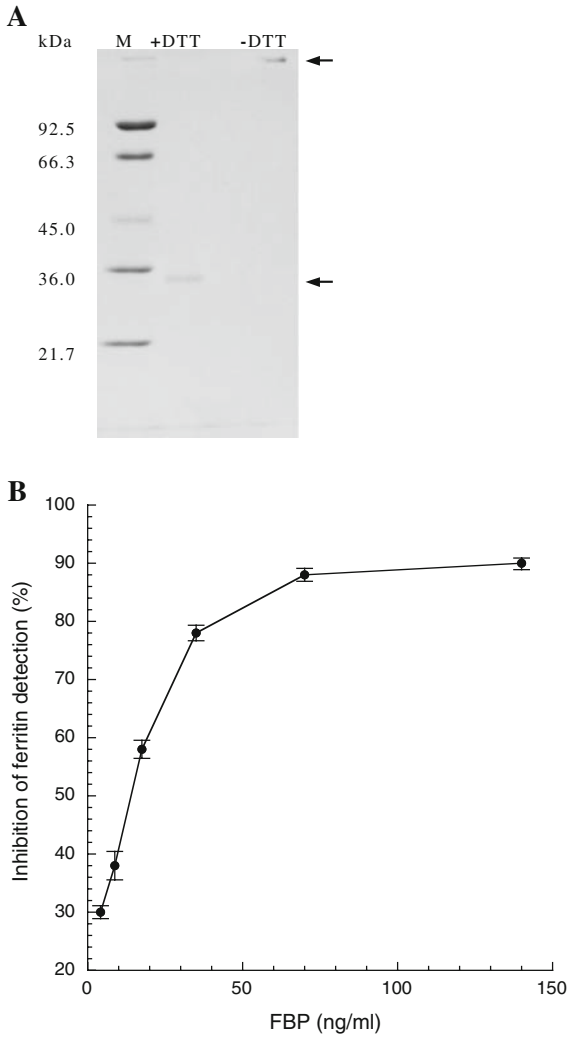

Fig. 4 Inhibition effect of novel FBP on the detection of canine liver ferritin. **A** SDS–PAGE of purified novel FBP. FBP (1 μg) was subjected to 10% SDS–PAGE in the presence or absence of 40 mM DTT. M: molecular mass marker proteins (2 μg each). Upper and lower bands indicate the novel FBP resolved under non-reducing and reducing conditions, respectively. **B** Known amounts of feline liver ferritin were added to a final concentration of 5 ng ml^{-1} to various amounts of novel FBP diluted with ELISA buffer containing 0.5 M $(NH_4)_2SO_4$ (pH 7.2), and the mixture was subjected to sandwich ELISA to detect ferritin. The value obtained in the absence of feline serum was defined as no inhibition. Each value is the mean ± SD of four replicates

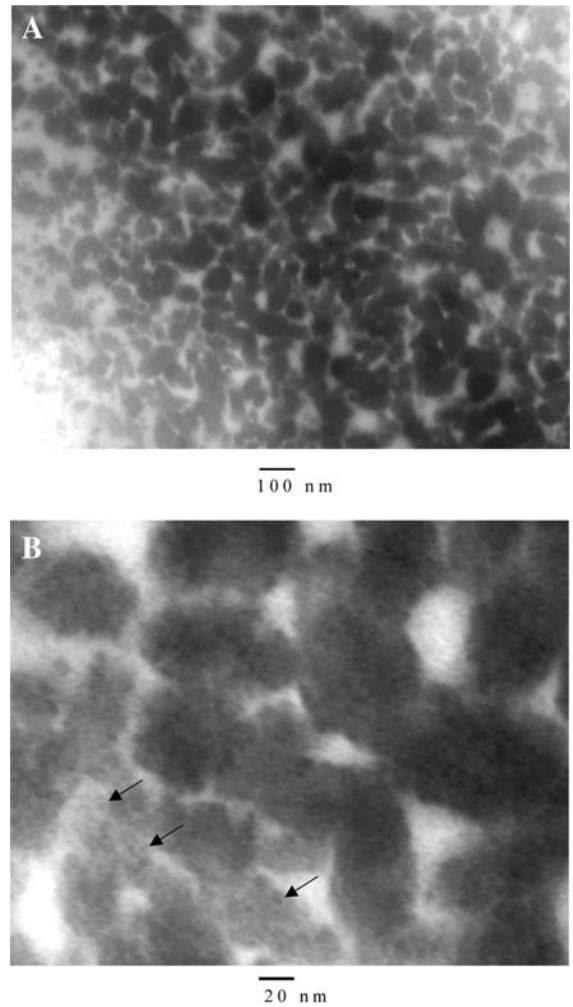

Fig. 5 The Electron micrograph of purified novel FBP, negatively stained with 2% phsphotangutate. Bars represent 100 nm and 20 nm in **A** and **B**, respectively. Arrows indicate a pentameric disk-like configuration

showed no protein band (Fig. 3C). After purification by electro-elution from a Native PAGE gel, FBP showed 31 kDa and 130 kDa bands in reducing and non-reducing conditions, respectively (Fig. 4A). Purified FBP inhibited detection of feline liver ferritin by immunoassay in a dose-dependent manner (Fig. 4B). A negative staining of the purified protein showed conglomerate of aggregated pentraxin-like molecules with a central pore and a diameter of 10 nm with 20,000 and 50,000-fold magnifications (Fig. 5A, B).

Discussion

Feline and canine liver ferritins used in this study had almost the same ferritin subunit H/L ratio (feline: 2.5; canine: 2.4). The amino acid sequences of H

(AB193257) and L (AB193258) feline ferritin showed remarkably high homology with those of H and L subunits of canine ferritin (Orino et al. 2005) (H: 98%; L: 96%). There was almost the same dose response in standards of feline and canine liver ferritins in the immunoassay used in this study (data not shown), suggesting that feline FBPs react with canine liver ferritin at the same level as with feline liver ferritin. As shown in Fig. 1B, feline serum remarkably inhibited the detection of canine liver ferritin. In fact, feline serum caused almost the same inhibition for feline and canine ferritin detection (data not shown). The inhibitory effects of horse and bovine serum on their ferritin immunoassays disappear after heat treatment (75°C, 15 min) (Orino et al. 1993a) or by increasing the ionic strength to 0.5 M $(NH_4)_2SO_4$ (Orino et al. 2004), respectively. Although Andrews et al. (1994) developed the feline serum ferritin immunoassay; they found no inhibitory effect of feline serum. However, in our sandwich ELISA using rabbit anti-rat liver ferritin antibody and biotin-labeling, we observed an inhibitory effect of feline serum on the ferritin immunoassay; this inhibitory effect was not eliminated even with heat treatment (75°C, 15 min) in the presence of 0.5 M $(NH_4)_2SO_4$ (data not shown). Therefore, we were not able to develop an immunoassay for feline serum ferritin. Although the reason for this difference between our results and those of Andrews et al. (1994) is not clear, it may be due to the difference in antibodies used because Andrews et al. (1994) used homogenous ferritin as the antigen for immunization to produce their ELISA antibody.

There are two types of FBPs, those that directly bind to ferritin and those that indirectly bind through heme (Parthasarathy et al. 2002; Orino et al. 1993b; Seki et al. 2008). Feline FBP inhibited both the detection of canine liver ferritin and its apoferritin, but its apoferritin showed weaker inhibition of ferritin detection than holoferritin. The binding of apoB with ferritin is heme-mediated, which is inhibited with an increase of ionic strength to 0.5 M $(NH_4)_2SO_4$, and only limited with holoferritin because iron removal from holoferritin via reduction simultaneously releases heme (Seki et al. 2008). Taken together, although 0.5 M $(NH_4)_2SO_4$ treatment completely abolished the immunoassay for apoferritin, we reached the preliminary conclusion that feline serum contains both types of FBPs.

We first tried to purify FBPs from feline serum using an affinity column packed with canine liver ferritin-coupled Sepharose 4B gel; FBPs were then eluted with PBS containing 0.5 M $(NH_4)_2SO_4$ followed by PBS containing 3 M KSCN. These eluted samples were incubated with canine liver ferritin, and their complexes were co-immunoprecitated with rabbit anti-bovine spleen antibody in place of rabbit anti-rat liver ferritin antibody, because anti-rat liver ferritin antibody abolishes the binding of canine serum FBPs, including anti-ferritin autoantibodies with canine liver ferritin, due to higher affinity for ferritin to the anti-ferritin antibody than canine FBPs (Orino et al. 2006b). Immunoblot analysis of the 0.5 M $(NH_4)_2SO_4$ eluate showed the presence of autoantibodies (IgG, IgM, and IgA) to ferritin with probes of antibodies specific for H chains of feline IgG, IgM, and IgA, although these antibodies cross-reacted with the H chain of rabbit IgG. Each H chain of feline IgG, IgM, and IgA migrated slower than the H chain of rabbit IgG in SDS–PAGE, although the H chain of feline IgG migrated only slightly slower. The 3 M KSCN eluate showed the same result as the 0.5 M $(NH_4)_2SO_4$ eluate according to immunoblot analysis (data not shown). Therefore, feline FBP was identified as anti-ferritin autoantibodies (IgM, IgG, and IgA) as in canine (Orino et al. 2006b), horse (Orino et al. 2006a), and bovine (Orino et al. 2004). We could not detect any effect of 3 M KSCN eluate on the ferritin immunoassay due to non-specific reactions causing binding to the well of the plate, followed by binding with biotinylated antibody for unknown reasons (data not shown). Therefore, although feline serum was found to contain anti-ferritin autoantibodies (IgG, IgM, and IgA), another factor, such as a novel FBP, may be involved in the inhibition of the ferritin detected by immunoassay.

We tried to purify the novel feline FBP that inhibits the ferritin immunoassay. Although a novel FBP was partially purified from feline serum with $(NH_4)_2SO_4$ fractionation (33–50%) and gel and ion exchange chromatography, we fully purified it by electro-elution from gel slices obtained by native PAGE of complexes formed between the FBP and Ft-Sepharose. SDS–PAGE of the novel FBP under both reducing and non-reducing conditions showed the same migration pattern, with unknown bands except for bands assumed to be ferritin subunits from complexes formed between partial purified FBP and

ferritin coupled-Sepharose 4B. Thus, the novel FBP was revealed to be a homomultimer composed of intermolecular disulfide bonds between the 31 kDa subunits. The novel FBP eluted faster than feline IgG (150 kDa) by Sephacryl S-300 gel filtration chromatography (data not shown). Therefore, the novel FBP was estimated to be a pentamer or a hexamer. In addition, the purified FBP inhibited detection of feline liver ferritin by immunoassay in a dose-dependent manner. This finding is the second reported case of an FBP inhibiting a ferritin immunoassay, the first being fibrinogen (Orino et al. 1993b).

Although the biochemical properties of the purified novel FBP are different from those reported in other animals, such as H-kininogen (Torti and Torti 1998), α-2-macroglobulin (Santambrogio and Massover 1989; Massover 1994), apoB (Seki et al. 2008), fibrinogen (Orino et al. 1993b), and anti-ferritin autoantibodies (Orino et al. 2004, 2006a,b), our attempt to identify the novel feline FBP was unsuccessful. The serum concentration of feline FBP was estimated to be approximately 20 μg ml^{-1} from the results shown in Figs. 1B and 4B. Although it is likely that the novel feline FBP is a serum amyloid P component (SAP), because it is a pentamer composed of 31 kDa subunits and is present at relatively high concentrations (10–30 μg ml^{-1}) in serum, the novel feline FBP showed intermolecular disulfide bonds and no binding with agarose or mannan in the presence of calcium, which is different from SAPs reported in other animals (Morimatsu et al. 1989; Laursen 2003) (data not shown). Feline SAP has not been purified yet. Amino acid sequence homologies among human (Mantzouranis et al. 1985), rat (Dowton and McGrew 1990), and mouse (Ishikawa et al. 1987) SAPs are high (69–79%); however, N-terminal amino acid analysis of the novel feline FBP was not successful due to unknown nonspecific cleavage. Further study is needed to identify the novel feline FBP by C-terminal amino acid analysis. However, a negative staining of the purified protein showed conglomerate of aggregated disk-shaped molecules with a central pore and a diameter of 10 nm with 20,000 and 50,000-fold magnifications. This sticky molecule seems to be a pentraxin-like molecule as described previously (Morimatsu et al. 1989). Although the purified protein is likely to be SAP, the protein sample was different in agarose-binding and non-sticky molecule as in SAP (Morimatsu et al. 1989). Expression of a feline SAP cDNA may lead to the identification of purified FBP.

In this study, feline FBP was identified as an autoantibody to ferritin. However, it is unlikely that the anti-ferritin autoantibody causes autoimmune disease by forming an immunocomplex with circulating ferritin. The developmental and immunoglobulin class changes of anti-ferritin autoantibodies in mammals have not yet been elucidated. In addition, we characterized the biochemical properties of the novel FBP, but further study is needed to reveal its sequence. The physiological roles of feline FBPs and their interactions with circulating ferritin remain to be clarified.

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