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Characterization of Ferritin Isolated from Dog Spleen

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Ferritin is known to be the principle iron-storage protein in a wide variety of organisms. The electrophoretic mobility and immunological cross-reactivity of dog splenic ferritin were compared with those of horse, bovine, and pig splenic ferritin after isolation using heat treatment, salting out, column chromatography, and ultrafiltration. These isolation methods allowed the recovery of ~84 μg of the ferritin per g of spleen. The iron content in the dog ferritin was 22.7%, which appeared to be higher than those in the other mammalian ferritins tested. The electrophoretic mobility of the dog ferritin under nondenaturing conditions was similar to its bovine counterpart, whereas it was more identical to pig and horse ferritins on an SDS-polyacrylamide gel. The molecular weight of the dog ferritin subunit was 19.5 kDa on an SDS-polyacrylamide gel, and the subunit was unable to bind with iron. The polyclonal anti-dog ferritin raised in rats was able to cross-react with the pig, bovine, and horse ferritins, upon Ouchterlony double immunodiffusion. A Western blot analysis also revealed that the anti-dog ferritin, which specifically bound with the dog ferritin subunit, could also recognize the horse, bovine, and pig ferritin subunits and the maximum cross-reactivity was exhibited with the pig ferritin subunit, indicating that the dog ferritin is immunochemically more similar to the pig ferritin than its other mammalian counterparts. Accordingly, these results elucidate the biochemical and immunochemical characteristics of dog ferritin that might have a potential to be applied as an oral iron supplement to treat iron deficiency anemia.

Key words – dog splenic ferritin, iron content, rat polyclonal anti-dog ferritin, immunological cross-reactivity

Iron plays an important role in living organisms, particularly as an essential element required for oxygen transport system in hemoglobin. Ferritin, which functions as an iron-storage protein or a sequestering protein for toxic free iron, has been found in a wide variety of organisms, including mammals[10], chickens[15,19], invertebrates[8,21], plants[24], fungi[6,18], and bacteria[23]. In mammals, the internal organs containing ferritin are spleen, liver, bone marrow, kidney, mucosal cells of small intestine, reticulocytes, placenta, testis, heart, and skeletal muscle[3,10]. Ferritin is a 460 kDa protein, which consists of 24 subunits, with a central core containing approximately 4,000~4,500 atoms of iron[9,12,20]. The characteristics for the ferritin subunit appear to vary depending on the source organism in that ferritin is composed of either chemically identical subunits or two different species heavy and light chains. While the molecular weight of the monomer subunit of bovine splenic ferritin is estimated to be about 18~19.5 kDa[2], the subunit of chicken liver ferritin is 22 kDa[15]. For horse splenic ferritin, the heavy chain is 21 kDa in molecular mass and the light chain is 19 kDa[1]. It has been suggested that these two subunit chains may be responsible for generating the structural heterogeneity of ferritin from different organs of the same species. However, although the physiological roles of ferritin with different heavy/light-chain ratio still remains to be elucidated, it has been reported that both chains complement each other in the process of sequestering and storing iron[9]. Iron absorbed from the outside is transported and stored in the ferritin based on the transferrin endocytosis system[5].

Anemia is a common medical disorder in the world and has been associated with several adverse health consequences including poor mental development, maternal mortality, and low-birthweight[13,16,17]. The main cause of anemia is known to be iron deficiency particularly in young children and pregnant woman, who are at increased risk due to their increased requirements. Since diet alone can not supply the iron required for treatment of the iron deficiency, the iron supplementation is routinely recommended for individuals who are at the high risk of iron deficiency. The treatment of iron deficiency in humans is usually accomplished through

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ingestion of oral iron supplements in dosages ranging up to about 120 mg/day[11]. To help oral iron intake, the ferritin purified from bovine and horse spleen had been made commercially available in several countries as a medicinal iron for the treatment of anemic conditions. In this context, horse spleen ferritin was distributed on the Korean market. However, the production of bovine and horse ferritin as a medicine for anemic conditions was discontinued due to a rising contamination-risk of contagious pathogens, such as the bovine spongiform encephalopathy virus and other pathogenic viruses related to horses. Therefore, the commercial supply of medicinal bovine and horse ferritin was also stopped in Korea.

Until now, most studies related with ferritin have focused on human, bovine, and horse ferritin with respect to the extraction, isolation, purification, molecular weight of native ferritin, molecular weight of the subunits, and crystallization[1-3,22]. Studies performed on ferritins from other origins have mainly focused on comparing their characteristics with the commercially available bovine and horse ferritin[8,15,24]. As such, an investigation of the biochemical and immunochemical characteristics of dog ferritin has not yet been reported. Accordingly, the present study attempted to purify dog splenic ferritin along with bovine and pig splenic ferritin to compare their iron contents and immunochemical characteristics, as an attempt to examine whether dog ferritin has a potential to be applicable to anemic conditions instead of bovine and horse ferritin. The results showed that the quantity of ferritin protein recovered was more abundant from the dog spleen, and the iron content in the dog ferritin was 22.7% (ratio of mg of ferrous iron per mg of ferritin protein), which was the highest iron content compared to those of the pig, bovine, and horse ferritins. In addition, although the dog ferritin was seemingly more similar to pig ferritin, it also appeared to share an antigenic identity with bovine, and horse ferritin. Therefore, these results suggest that dog splenic ferritin may be a potential remedy for anemic conditions as a substitute for bovine and horse ferritin.

MATERIALS AND METHODS

Chemicals, reagents, and animals

All the chemicals and reagents, including the horse ferritin, were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. The agarose was purchased from Bethesda Research Labratories (Bethesda, MD, USA). To obtain spleen ferritin, healthy adult animals, such as dogs, cows, and pigs were used. Healthy adult rats were also used to obtain liver ferritin.

Sepharose 6B gel permeation chromatography

The isolation of the splenic ferritins was performed essentially as previously described[2,3,8]. Fresh spleens obtained from individual adult animals, such as dogs, pigs, and cows, were cut into small pieces (about 1~3 g) and mixed with distilled water (1.5 ml/g of spleen) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The mixture was ground with a blender and heated for 10 min at 70℃ in a water bath. After centrifugation at 2,500 g for 30 min, the supernatant was harvested, and then the pH was adjusted to 4.8. After incubation for 20 h at 4℃, the solution was centrifuged at 8,000 g for 30 min and the precipitate was discarded. The supernatant was adjusted to pH 6.5, and β-mercaptoethanol was added to a final concentration of 0.02%. Next, ammonium sulfate was added to create 75% saturation, and then the solution was incubated at 4℃ for 20 h. Thereafter, the solution was centrifuged at 2,500 g for 30 min, and then the precipitate was harvested and was dissolved in distilled water containing 0.1 mM PMSF and 0.02% β-mercaptoethanol. Again, the solution was adjusted to a 75% saturation of ammonium sulfate. The precipitate was then dissolved in distilled water and dialyzed against 0.15 M NaCl at 4℃. This final spleen extract was concentrated by ultrafiltration to a 30 kDa average molecular weight cutoff.

The ferritin extract was applied on a Sepharose 6B gel filtration column (26×950 mm), which had been pre-equilibrated with a 0.05 M borate buffer (pH 8.6) containing 0.01 M KCl. The column was eluted with the same buffer solution at a flow rate 15 ml per h and the eluent fractionated into 5 ml portions.

Electrophoresis of ferritin under nondenaturing or denaturing condition

The nondenaturing polyacrylamide gel electrophoresis was performed using a 6% polyacrylamide gel without sodium dodecylsulfate (SDS). The conditions were as follows: Tris-Glycine electrophoresis buffer, pH 8.3, current of 20 mA, and 15 μg of ferritin protein per lane. The cellulose acetate strip electrophoresis was carried out using a barbital buffer (pH 8.6) at 200 V. After electrophoresis, the gel or strip

was stained with either 0.5% ponceau S in 5% sulfosalicylic acid to detect the ferritin protein or 0.5% potassium ferrocyanide in 1 N HCl to measure the iron.

To investigate the molecular weight of ferritin subunit under denaturing conditions, the ferritin protein was subjected to 15% SDS-polyacryamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was stained with a 0.1% comassie brilliant blue R250 solution to detect the protein bands or 0.5% potassium ferrocyanide in 1 N HCl to measure the iron as previously described[2]. The molecular weight of ferritin subunit was estimated by comparing its relative mobility with those of standard proteins (SeeBlue Plus2 Pre-stained Standard, Invitrogen, Carlsbad, CA, USA).

Assay of protein and iron

Quantification of the protein was performed using the BCA assay method (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. To content of ferrous iron in the ferritin was determined using AOAC Official Methods of Analysis with a modification[4]. Briefly, after the ferritin sample mixed with sulfuric and nitric acids was boiled and chilled, the protein shell was removed. Sequentially, the solution was then mixed with an acetate buffer (pH 4.8), ascorbic acid, and 2,2'-dipyridyl. The iron was measured using a spectrophotometer at 521 nm after forming a coloured complex developed by the presence of iron. The quantity of iron in the ferritin was calculated by using ferrous ammonium sulfate as a standard.

Immunization of rat with the dog splenic ferritin

The polyclonal antibody was raised in eight male rats (300 g) based on subcutaneous injections at five sites with 200 μg of dog splenic ferritin, emulsified with Complete Freund's Adjuvant (Sigma-Adrich, St. Louis, MO, USA) in a ratio of 3:1. After two weeks, a test bleeding was performed from the tail. For the secondary immunization, 200 μg of dog splenic ferritin mixed with Incomplete Freund's Adjuvant was injected with the same manner as the first injection. Then four weeks after the first injection, the bleeding was carried out by heart-puncture and the antiserum was obtained from the blood.

Ouchterlony double immunodiffusion

To investigate the interaction between the antibody and a specific antigen, Ouchterlony double immunodiffusion were carried out in duplicate in a 1% (w/v) agarose gel containing a 0.05 M barbital buffer (pH 8.6). The precipitin reaction in the gel was allowed to continue for 48 h at 37℃ in a humid chamber. The agarose plate was then washed in 1 M sodium chloride to remove any non-reacted protein. Next, one gel was stained with 0.5% ponceau S in 5% sulfosalicylic acid to better visualize the precipitin lines between the anti-ferritin and the ferritin, while the other gel with 0.5% potassium ferrocyanide in 1 N HCl to measure the iron.

Western blot analysis

For the Western blot analysis, 10 μg of the purified ferritin protein samples was electrophoresed on a 15% SDS-polyacrylamide gel and electrotransferred to an Immobilon-P membrane (Millipore, Bedfrod, MA, USA), as described elsewhere[14]. The membrane was then allowed to react with the anti-ferritin antiserum diluted 2000-fold in a blocking buffer (3% non-fat skim milk, 50 mM Tris, 10 mM NaCl, 1% Tween 20, pH 7.4) and horse-radish peroxidase conjugated sheep anti-rat antibody. The detection of the ferritin protein was visualized on X-ray film using an ECL Western blotting detection system (Amersam Pharmacia Biotech, UK) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Isolation and purification of the dog splenic ferritin

To fractionate the spleen extract containing ferritin by gel permeation chromatography, the final concentrate was applied to a column of Sepharose 6B and the ferritin was eluted with a 0.05 M borate buffer (pH 8.6) supplemented with 0.01 M KCl (Fig. 1A, B, and C). The individual fractions corresponding to the protein peaks were analyzed by 4~20% gradient SDS-PAGE, along with commercially available horse splenic ferritin (Sigma, St. Louis, MO, USA) as an authentic marker (Fig. 1D, E, and F). When the electrophoretic patterns of the fractions corresponding to the individual protein peaks were compared with that of the horse ferritin, the fractions expected to be ferritin were between the number 50 and 65 (50~65) for the dog spleen extract, 34~59 for the pig spleen extract, and 34~46 for the bovine spleen extract. The fractions that appeared to contain ferritin were combined and concentrated by ultrafiltration. Rat ferritin was also isolated from a liver extract in the same manner (data not shown).

Electrophoretic mobility of dog splenic ferritin

To confirm whether the fractions collected from the

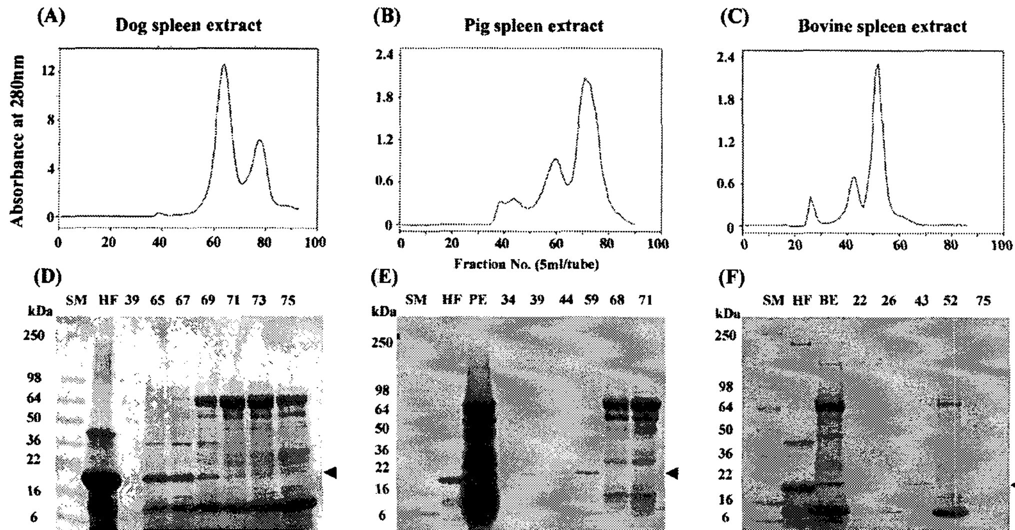

Fig. 1. Fractionation of dog (A), pig (B), and bovine (C) spleen extracts on Sepharose 6B column (26×950 nm) chromatography and resolution of individual protein peak fractions together with commercially available horse splenic ferritin on 4~20% gradient SDS-PAGE (D, E, and F). The arrow head indicates the location of ferritin. Symbols: SM, protein size marker; HF, commercial horse splenic ferritin; PE, pig spleen extract; BE, bovine spleen extract.

Sepharose 6B column chromatography contained ferritin and also examine their purities, the collected ferritin fractions were resolved on a 6% polyacrylamide gel under nondenaturing conditions. After electrophoresis, the gel was stained with either ponceau S to detect protein bands or potassium ferrocyanide to detect irons. As shown in Figs. 2A and B, all the major protein bands in each lane stained with ponceau S also turned out to contain iron after being stained with potassium ferrocyanide. These results indicated that the collected fractions were mainly ferritin proteins bound with iron. The purity of these collected ferritin fractions was also determined when the fractions were resolved on cellulose acetate strip electrophoresis (Fig. 2C and D). In the bovine, dog, and pig splenic ferritin fractions as well as the commercially available horse ferritin, at least one minor band was detectable on the 6% polyacrylamide gel electrophoresis. However, when the electrophoresis was performed on a cellulose acetate strip, all ferritin fractions exhibited one major band without any minor bands, except the pig splenic ferritin fraction, which appeared to possess an additional minor band. Since the difference between the polyacrylamide gel and the cellulose acetate strip electrophoresis in determining the resolution was mainly the pore size of the matrix, it would seem likely that the cellulose acetate strip had relatively larger pores than the 6% polyacrylamide gel and thus hardly exerted any sieving effect on the electrophoretic mobility of proteins. Accordingly, under these nondenaturing conditions, the electrophoretic

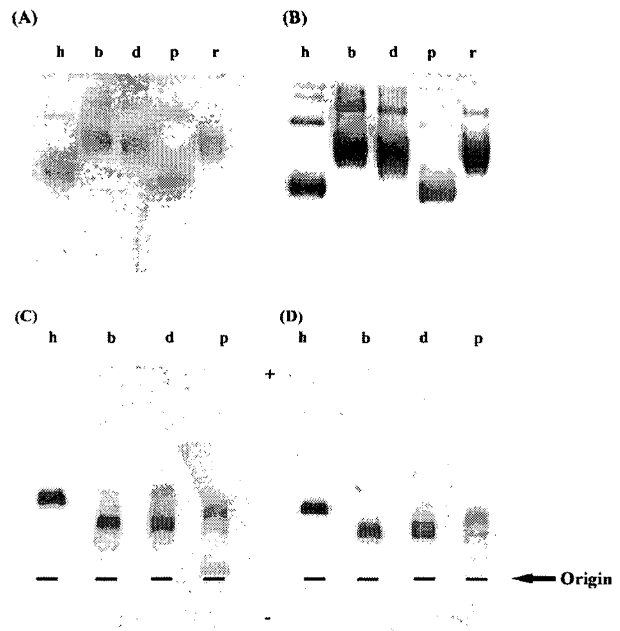

Fig. 2. Electrophoretic patterns of horse (h), bovine (b), dog (d), pig (p), and rat (r) ferritin samples on 6% polyacrylamide gel (A and B) and cellulose acetate strip (C and D) under nondenaturing conditions. The electrophoresis of 15 μg of ferritin protein was performed in duplicate, and then one was stained with ponceau S to detect protein (A and C), while the other was treated with potassium ferrocyanide to detect the presence of iron (B and D).

mobility of the dog and bovine splenic ferritin appeared to be similar and exhibited a slower mobility to the anode than their horse and pig counterparts.

Molecular weight of dog splenic ferritin subunit as determined by SDS-PAGE

To measure the molecular weight of the ferritin subunit, electrophoresis was performed on 15% SDS-polyacrylamide gel under denaturing conditions. After the electrophoresis was completed on duplicate gels, one gel was stained with comassie brilliant blue to detect protein bands, while the other gel was treated with potassium ferrocyanide to detect irons. The major bands in each lane were the subunits of the ferritin. The molecular mass of the individual ferritin subunits was estimated based on the electrophoretic mobility of standard molecular markers. The molecular masses of the individual ferritin subunits were estimated to be approximately 19.5 kDa. for the dog ferritin, 18.8~21 kDa for the horse ferritin, 23.5 kDa for the bovine ferritin, 21 kDa for the pig ferritin, and 20 kDa and 14 kDa for the rat liver ferritin (Fig. 3A). Under these denaturing conditions, the electrophoretic pattern of the dog ferritin appeared to be more similar to those of the horse and pig ferritin as compared to the bovine ferritin. None of the ferritin subunits was able to bind with iron in a monomer state (Fig. 3B). Instead, it is likely that some of the protein bands with small molecular weights were still bound with iron. The origin of these proteins still remains unclear. Although it has been reported that horse splenic ferritin is composed of a heavy chain of 21 kDa and light chain of 19 kDa[1], in the current study these two monomer subunits were not clearly detectable from the commercially available horse splenic ferritin but rather appeared to be a broad band within a range of 18.8~21 kDa. In addition, the subunit of the bovine splenic ferritin, detected as having a 23.5 kDa molecular mass in the current study, was slightly different from a previous report where the molecular weight was approximately 18~19.5 kDa[2].

Quantification of the iron content in the dog splenic ferritin

When the quantities of the ferritin proteins recovered from 1 g of dog spleen, bovine spleen, and pig spleen were measured, there were significant differences between the species. While the amount of ferritin protein obtained from 1 g of dog spleen was 84.0 μg, the amounts from the bovine and pig spleens were 46.8 and 16.0 μg, respectively. These results suggest that the content of the ferritin protein in the spleen might be different between dog, bovine, and pig. In addition, the content of ferrous iron in 1 mg of dog,

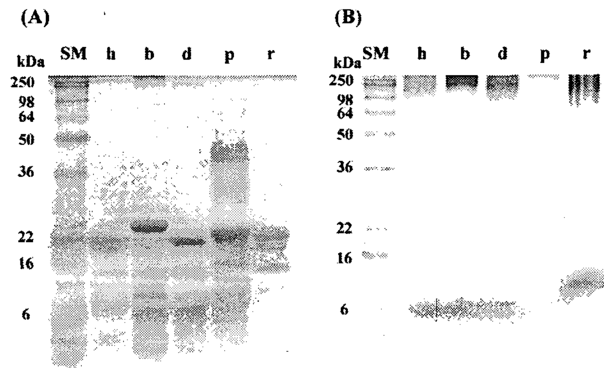

Fig. 3. Determination of molecular weight of horse (h), bovine (b), dog (d), pig (p), and rat (r) ferritins on 15% SDS-PAGE under denaturing conditions. The electrophoresis was performed in duplicate, then one was stained with ponceau S to detect proteins (A), while the other was treated with potassium ferrocyanide to detect the presence of iron (B).

bovine, pig, and horse splenic ferritin was 22.7, 20.2, 7.2, and 18.5%, respectively. These results indicate that although the iron level in the individual splenic ferritin proteins from dog, bovine, and horse appeared to be similar, it was actually highest in the dog splenic ferritin. The lowest level of iron content was found in the pig splenic ferritin when compared to the dog, horse, and bovine ferritin. This result was consistently observed, because the pig splenic ferritin band exhibited the faintest colour when the ferritin proteins were stained with potassium ferrocyanide following electrophoresis under nondenaturing conditions (Fig. 2B and D). Recently, for the treatment of iron deficiency in humans, oral iron supplementation in dosages ranging up to about 120 mg/day has been recommended[11]. It is also noteworthy that while the amount of iron needed to be absorbed for woman during the latter stage of pregnant is only 4~6 mg/day, oral iron supplement in dosages of 30~40 mg/day is required to be ingested due to the inefficient absorption of orally administrated iron[17]. Since the recovery level from the spleen and the iron content were highest in the dog splenic ferritin among the related ferritins tested, the dog splenic ferritin was expected to possess better potential than related ferritins from other species to be used as an oral iron supplement.

Comparison of dog splenic ferritin with related ferritins from other sources using Ouchterlony double immunodiffusion as well as Western blot analysis

To examine whether the dog splenic ferritin shared

antigenic determinants (also called epitopes) with related bovine, horse, and pig ferritins, Ouchterlony double immunodiffusion was performed using antiserum against dog splenic ferritin raised in rats. As shown in Fig. 4A, a single precipitin line was formed between antiserum (center) and two adjacent dog splenic ferritins (d) fused to give a single curved line of identity. This demonstrates that the antiserum specifically recognized and bound with its antigen, the dog splenic ferritin. All the precipitin lines formed between two adjacent antigens of dog splenic ferritin and bovine splenic ferritin (b), two adjacent antigens of dog splenic ferritin and pig splenic ferritin (p), or two adjacent antigens of dog splenic ferritin and horse splenic ferritin (h) against the antiserum in the center exhibited a typical line of identity as well as a curved spur form (Fig. 4C).

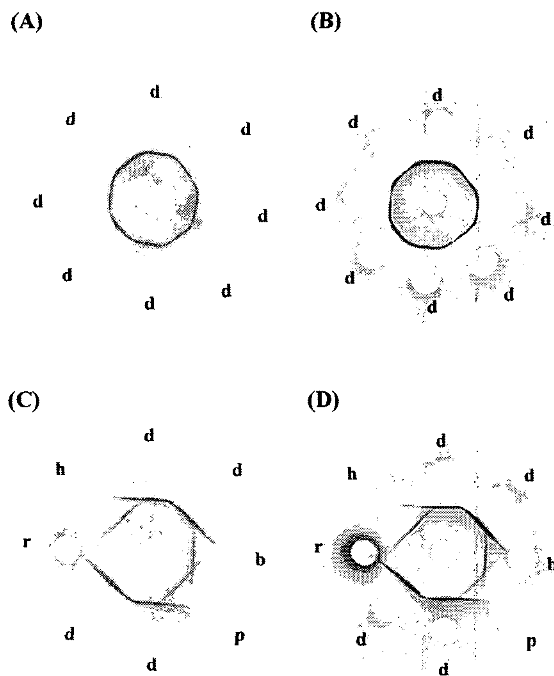

Fig. 4. Comparison of immunochemical characteristics of dog splenic ferritin with those of horse, bovine, pig, and rat ferritin using Ochterlony double immunodiffusion method. The Ochterlony double immunodiffusion was performed in duplicate in a 1% agarose gel supplemented with a 0.05 M barbital buffer (pH 8.6). The well in the center of the gel contained the rat polyclonal anti-dog splenic ferritin antibody, while the wells labeled d, b, p, r, and h contained the dog, bovine, pig, rat, and horse splenic ferritins, respectively. The precipitin reaction in the gel was allowed to continue for 48 h at 37℃. One gel was stained with ponceau S to better visualize the precipitin (A and C), while the other gel was stained with potassium ferrocyanide to measure the iron (B and D).

These results indicate that these ferritins shared partial identity with dog splenic ferritin in their antigenicity such that the anti-dog splenic ferritin could recognize these related ferritins from other sources by the cross-reactivity. However, when precipitation reaction was induced between the antiserum (center) and two adjacent antigens of bovine (b) and pig splenic ferritin (p), the antiserum produced an independent precipitin line with each antigen, and the two lines became crossed. This shows that the two antigens did not share common epitopes that could be recognized by the anti-dog splenic ferritin. As shown in Fig. 4B and D, the precipitin line formed upon Ouchterlony double immunodiffusion was stainable with potassium ferrocyanide. This confirms that the precipitin reaction specifically occurred between the antiserum and ferritin.

To identify the cross-reaction of the antiserum raised against dog splenic ferritin with that of the other species, a Western blot analysis was performed. As shown in Fig. 5, the dog splenic ferritin subunit with molecular weight of 19.5 kDa and the pig ferritin with a similar molecular weight were both well detected. In addition, the bovine splenic ferritin subunit of 23.5 kDa as well as the horse splenic ferritin subunit of 19.0 kDa was also detected by the antiserum as a faint band. However, the rat ferritin subunit

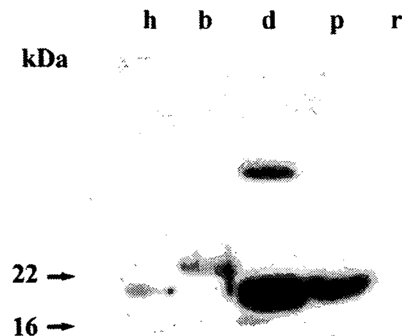

Fig. 5. Western blot analysis of horse (h), bovine (b), dog (d), and pig (p) splenic ferritin, and rat (r) liver ferritin using rat polyclonal anti-dog antibody. Equivalent amounts of the individual ferritin proteins were subjected to 15% SDS-PAGE and electrotransferred to an Immobilon-P membrane. The membrane was probed with a 1:2000 dilution of the rat polyclonal anti-dog ferritin, then the detection of ferritin protein was visualized on X-ray film using an ECL Western blotting detection system, as described in Material and Methods.

was not detected by the antiserum as expected, because the anti-dog ferritin was raised in rats. These results indicate that the dog splenic ferritin had the highest antigenic identity with the pig splenic ferritin, yet the lowest identity with the bovine and horse splenic ferritins among the related ferritins tested. In summary, it was demonstrated that dog splenic ferritin could be easily isolated using biochemical methods, such as heat treatment, salting out, column chromatography, and ultrafiltration, and that both the recovery level from the spleen and the iron content were highest in the dog splenic ferritin among the related ferritins tested. Accordingly, the current results suggest that dog splenic ferritin may have greater potential than related ferritins from other species to be developed as a remedy for anemic conditions.

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초록 : 개의 비장에서 분리한 페리틴의 특성

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페리틴은 생체 내 주요 철 저장 단백질로서 포유류에서 세균류에 이르기까지 다양한 생명체에 존재한다. 페리틴 분자는 18~22 kDa의 단량체 24개가 결합된 약 240 kDa 분자량을 지닌 거대분자이다. 본 연구에서는 개의 비장에서부터 열처리, 염석, 컬럼 크로마토그래피, 그리고 한외여과 등의 방법으로 페리틴을 정제한 후, 그 전기영동상의 특성 및 면역화학적 특성을 말, 소, 돼지 등의 비장 유래 페리틴과 비교 분석하였다. 이러한 정제방법에 의해 개의 비장으로부터 페리틴의 양은 비장 1 g당 약 84 μg이었다. 정제된 개 페리틴의 철 함량은 22.7%로서 함께 비교 검토한 다른 동물 유래의 페리틴에 비해 가장 높게 나타났다. 개 페리틴의 비변성 겔에서의 이동상은 소 페리틴과 유사하였고, 변성 겔에서는 돼지 및 말의 페리틴과 유사하였다. SDS-PAGE상에서 나타난 개 페리틴 subunit의 분자량은 19.5 kDa이였으며 이때 SDS-PAGE상의 ferritin subunit은 철과 결합하지 않는 것으로 확인되었다. 개 페리틴의 면역화학적 특성을 다른 동물유래의 페리틴과 비교하고자, 개의 페리틴에 대한 다클론 항체를 쥐에서 생산하였다. 생산된 항-페리틴 항체를 이용하여 Ouchterlony doulbe immunodiffusion 방법으로 개, 소, 말, 돼지 페리틴들을 항원으로 항원-항체반응을 조사한 결과, 항-개 페리틴 항체가 소, 말, 돼지의 페리틴과도 항원-항체반응을 일으킬 수 있는 것으로 나타났다. 이는 개, 소, 말, 돼지 페리틴들이 항원적 동일성을 지니고 있음을 시사한다. 항-개 페리틴 항체를 이용하여 타 동물 유래의 페리틴에 대한 Western blot analysis를 시도한 결과, 항-개 페리틴 항체가 개 페리틴 및 돼지 페리틴에는 강하게 반응하였으나 말과 소 유래의 페리틴에 대해서는 비교적 약하게 반응하여, 개의 페리틴이 면역화학적으로 돼지 페리틴과 가장 유사한 것으로 나타났다. 이상의 결과들은 개의 페리틴에 대한 생화학적 및 면역학적 특성을 제시한다.