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Purification of chicken liver ferritin by two novel methods and structural comparison with horse spleen ferritin

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Ferritin was purified from chicken liver by two different methods: gel filtration on controlled-pore glass beads, and immunoaffinity chromatography employing a chicken ferritin-specific monoclonal antibody that did not cross-react with horse spleen ferritin. This antibody recognizes intact ferritin and an oligomeric 240 kDa form of the molecule after protein transfer to nitrocellulose, but not the 22 kDa chicken ferritin subunit. Chicken liver ferritin purified by these methods exhibited reduced migration on non-denaturing polyacrylamide gels compared with horse spleen ferritin. These results were consistent with the difference in calculated isoelectric points of chicken and horse ferritin subunits. By two-dimensional gel electrophoresis, chicken ferritin 22 kDa subunits exhibited isoelectric points from 6.1 to 6.6 whereas horse spleen ferritin subunits exhibited isoelectric points of 5.8–6.3. The 240 kDa form of the chicken ferritin molecule had an isoelectric point of 6.6 whereas the 210 kDa form of the horse ferritin molecule had isoelectric points of 5.1 and 4.9. Intact chicken liver ferritin particles were 13.4 ± 0.8 nm (controlled-pore glass-purified) and 12.5 ± 0.9 nm (affinity-purified) in diameter when viewed by electron microscopy. Horse spleen ferritin consisted of slightly smaller particles with an average diameter of 11.0 ± 0.7 nm. However, ferritin from chicken liver and horse spleen co-migrated with an apparent molecular mass of 470 kDa when analysed by Sepharose 4B gel filtration chromatography. These results indicate that, consistent with results from other published purification methods, the chicken ferritin purified by the methods reported here exhibits both structural similarities to, and differences from, horse spleen ferritin.

INTRODUCTION

Ferritin is the major iron-storage protein found in vertebrates (Laufberger, 1937), invertebrates (Towe *et al.*, 1963), plants (Hyde *et al.*, 1963), fungi (David & Easterbrook, 1971), and bacteria (Yariv *et al.*, 1981). As such, it has been extensively studied and its protein structure characterized in some of these organisms (Rice *et al.*, 1983).

Because of the large amount of ferritin present in horse spleen, this has been the primary source of ferritin used for structural and electron microscopic localization studies. The molecular mass of horse ferritin varies depending on the organ source. It has a molecular mass of 460 kDa when isolated from spleen and 515 kDa when isolated from heart. The apoferritin consists of 24 subunits of two types designated H (21 kDa) and L (19 kDa) which are responsible for generating the structural heterogeneity of ferritin from different organs of the same species (Drysdale, 1977; Arosio *et al.*, 1978). Human ferritin displays similar properties (Otsuka *et al.*, 1981). Despite relatively similar molecular masses for ferritins from various sources, there are clear species-specific differences which are readily detected by immunological methods (Addison *et al.*, 1984).

Chicken spleen ferritin has been characterized and its structure compared with horse ferritin (Shinjyo, 1973). More recently, the partial purification (Gonzalez del Barrio & Martin-Mateo, 1983) and the purification (Santos-Benito & Martin-Mateo, 1983) of chicken liver ferritin were also reported. In this report we describe the purification of chicken liver ferritin by two novel methods: gel permeation chromatography over controlled-pore glass beads, and immunoaffinity chromatography using a monoclonal antibody specific for chicken ferritin. We then used polyacrylamide-gel electrophoresis (PAGE), isoelectric focusing, electron microscopy and gel filtration chromatography techniques to verify the identity of the chicken ferritin and to compare its structure with the known structural characteristics of horse spleen ferritin. We show that, as expected, chicken liver ferritin purifed by these methods is structurally different from horse spleen ferritin.

EXPERIMENTAL

Materials

Horse spleen was purchased from Miles Laboratories, Elkhart, IN, U.S.A.; controlled-pore glass beads from Electro-Nucleonics Inc., Fairfield, NJ, U.S.A.; CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals; ultra-pure urea from Schwarz–Mann, Orangeburg, NY, U.S.A.; acrylamide, SDS and other electrophoresis reagents from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Ampholines from LKB and CHAPS from Sigma

Abbreviations used: CB, column buffer (10 mM-Hepes, pH 7.0/10 mM-EGTA/0.2 M-sucrose/0.3 M-NaCl/0.02% NaN_3); IB, isolation buffer (0.1 M-Mes, pH 6.5/1 mM-EGTA/1 mM-$MgCl_2$/0.02% NaN_3; SDS buffer, 0.125 M-Tris, pH 6.8/2% SDS/20% glycerol/15 mM-dithiothreitol; PAGE, polyacrylamide-gel electrophoresis; PEG, poly(ethylene glycol); CPG, controlled-pore glass.

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Chemical Company or Calbiochem. All other chemicals were of reagent grade.

Purification of chicken liver ferritin by gel permeation chromatography

All procedures were carried out at 4 °C, unless otherwise stated. Rooster livers obtained from a local slaughterhouse were minced on ice with single-edged razor blades. A Waring Blendor was used to homogenize 300 g of tissue with three 30 s bursts of isolation buffer (IB). After centrifuging this homogenate at 20000 *g* for 50 min, the pellets were discarded and the supernatants (chicken liver extract) were centrifuged at 100000 *g* for 1 h. The resultant microsomal pellets were resuspended in 300 ml of IB and processed according to the method of Nandi *et al.* (1982). Briefly, microsomes were centrifuged at 20000 *g* for 20 min to remove aggregates and the supernatants were again centrifuged at 100000 *g* for 1 h. This low-speed/high-speed centrifugation was repeated with the resuspended pellets. The high-speed supernatants were combined and used for the purification of ferritin by antibody affinity chromatography (see below). The final pellets were resuspended in 18 ml of IB, divided into three equal parts and layered onto three Beckman Ti60 centrifuge tubes containing 8 % sucrose in IB prepared in ^2H_2O. After centrifugation at 80000 *g* for 2 h, the pellets containing ferritin, coated vesicles and uncoated membrane vesicles were resuspended in IB containing 10 % sucrose and centrifuged at 20000 *g* for 20 min to remove aggregates. Part of the resulting supernatants was applied to a 1.5 cm × 100 cm column containing controlled-pore glass beads of average diam. 190 nm (Pfeffer & Kelly, 1981; Kelly *et al.*, 1983). The column was equilibrated and eluted with column buffer (CB). After several uses, glass beads were re-generated with 10-mM urea at 37 °C for 5 min and washed five times with deionized water. The beads were then re-treated with 1 % poly(ethylene glycol) (PEG)-20000 that had been filtered at 24 °C through successive membrane filters of 5.0 μm, 1.2 μm, and 0.45 μm pore size. About 500 ml of PEG-20000 was added to 150 ml of beads and stirred by gentle shaking for 20 min. After washing five times in deionized water and degassing, the beads were washed with CB and applied in CB to the column by vortex-mixing.

Antibodies

Monoclonal antibody recognizing chicken ferritin was prepared in collaboration with Dr. D. Fambrough, Dept. of Biology, Johns Hopkins University, Baltimore, MD, essentially as described (Fambrough & Bayne, 1983) using chicken liver coated-vesicle preparations as the immunogen. Purified anti-ferritin IgG was obtained by using DEAE chromatography to isolate IgG from mouse ascites fluid. Rabbit anti-(mouse IgG) coupled to horseradish peroxidase (RAM-HRP) was obtained from Sigma Chemical Company.

Purification of chicken ferritin by antibody affinity chromatography

Monoclonal anti-ferritin IgG was coupled to CNBr-activated Sepharose 4B using established procedures (van Eijk & van Noort, 1976). This anti-(chicken ferritin) monoclonal antibody IgG coupled to Sepharose was used to prepare an affinity column by packing a polypropylene 5 ml pipette with 3.5 ml of beads containing 2.0 mg of purified IgG. The column was equilibrated with IB at pH 6.5 at a flow rate of 5 ml/h. Either soluble chicken liver supernatants (see above) or Triton X-100-soluble membrane pellets were applied to the affinity column at a flow rate of 5 ml/h. After the sample had been applied, the column was washed with 20 bed vol. of IB followed by IB containing 0.1 % Triton X-100 and 0.5 M-NaCl. Bound protein was eluted with 10 ml of freshly-prepared 0.1 M-triethylamine, pH 11.0, at a flow rate of 10 ml/h. Samples (0.5 ml) were collected in tubes containing 0.5 ml of 1 M-sodium phosphate, pH 6.8, to neutralize the triethylamine. The column was re-equilibrated with IB at pH 6.5 and kept at 4 °C for further use. The highly-purified ferritin eluted from the column was concentrated and desalted on an Amicon ultrafiltration apparatus equipped with a PM-10 filter.

Enzyme-linked immunoassays (e.l.i.s.a.)

Chicken liver ferritin was detected in fractions from the gel permeation chromatography column by a direct-binding e.l.i.s.a. as described (Kelly *et al.*, 1983). A competitive e.l.i.s.a. was used to quantify the degree of cross-reactivity of the anti-(chicken ferritin) antibody with horse spleen ferritin and other heterologous proteins (Kelly *et al.*, 1983).

Polyacrylamide-gel electrophoresis

Two-dimensional gel electrophoresis was essentially as described by O'Farrell (1975) except that gel solutions contained 2 % CHAPS detergent instead of Triton X-100 (Perdew *et al.*, 1983). Gradient 3.75–15 % acrylamide slab gels were prepared according to the method of Laemmli (1970) and stained with either Coomassie Brilliant Blue at 0.1 % in 40 % methanol/10 % acetic acid and/or with silver using the procedure of Merril *et al.* (1981).

Non-denaturing polyacrylamide-gel electrophoresis (PAGE) was performed exactly as described above except that SDS was not present in the acrylamide gels or the electrode buffers.

Electrophoretic transfer of protein to nitrocellulose

Ferritin was purified by immunoaffinity chromatography and resolved on 3.75–15 % acrylamide gels (SDS/PAGE) after heating the sample at 100 °C or at 24 °C for 5 min. Gels were transferred to nitrocellulose according to the method of Towbin *et al.* (1979).

Sepharose 4B gel filtration chromatography

The molecular mass of chicken liver ferritin was estimated by co-elution of ^{125}I-labelled chicken ferritin and unlabelled horse spleen ferritin (470 kDa). Thyroglobulin (669 kDa) and catalase (235 kDa) were chromatographed separately to calibrate the column. Vitamin B_{12} (1450 Da) was used to estimate the included volume of the column. Chicken ferritin was iodinated by the Iodogen bead method (Pierce Chemicals) by incubating 100 μCi of Na^{125}I with 35 μg of chicken liver ferritin purified by either affinity chromatography or gel permeation chromatography.

Electron microscopy

Copper grids (400-mesh) were coated with Formvar and air-dried. A light coat of carbon was vacuum-evaporated on to the Formvar-coated grids and a drop of sample was applied. Samples containing sucrose eluted from the glass-bead chromatographic step were rinsed

with deionized water. Uranyl acetate (2%) was freshly prepared and centrifuged, and a drop of clear supernatant was applied to the sample on the grids. Samples were viewed and photographed on a Hitachi H-600 electron microscope at 75 kV.

RESULTS

Chicken liver ferritin was purified by controlled-pore glass chromatography as described in the Experimental section from a liver homogenate enriched for coated vesicles. The coated-vesicle fraction contained several polypeptides when analysed by SDS/PAGE (Fig. 1*b*). Some of these such as the 180 kDa, the 110 kDa, the 32–36 kDa (Woodward & Roth, 1978; Pearse & Bretscher, 1981), and the 53–56 kDa (Kelly *et al.*, 1983) proteins are common components of coated vesicles. However, the chicken liver preparation (Fig. 1*b*, lane a) contained an additional protein of apparent molecular mass 22 kDa which was not evident in bovine brain coated vesicles (Fig. 1*b*, lane b). In an attempt to further purify the 22 kDa protein, the chicken liver sample was applied to a controlled-pore glass-bead column which separates particles on the basis of size (Pfeffer & Kelly, 1981; Kelly *et al.*, 1983). Fractions collected from this column (Fig. 1*a*) were analysed for protein and membrane vesicle light scattering by absorbance at 280 nm, for protein by absorbance at 595 nm (Bradford, 1976), and for chicken ferritin by an enzyme-linked immunoassay using purified monoclonal antibody IgG by measuring the absorbance at 490 nm. Three peaks of absorbance at 280 nm were evident (Fig. 1*a*). The first peak, which contained very little protein, was eluted at the void volume of the column and when viewed in the electron microscope contained lipid vesicles (results not shown). The second peak contained most of the protein in the preparation and some anti-(chicken ferritin) binding activity. The third peak showed substantial anti-ferritin antibody binding. The absorption coefficient for the peak III fractions 90–110 was 35 ($A^{1\%}_{280}$) which is close to the absorption coefficient calculated for horse spleen ferritin ($A^{1\%}_{280} = 34$). When samples were electrophoresed on SDS/PAGE (Fig. 1*b*), this third peak contained a major band migrating at an apparent molecular mass of 22 kDa, characteristic of the ferritin subunit molecular mass.

To verify that the 22 kDa polypeptide was a ferritin subunit, we also purified chicken ferritin from liver by a different method employing immunoaffinity chromatography using a specific anti-ferritin monoclonal antibody. This anti-(chicken ferritin) monoclonal antibody does not cross-react with horse spleen ferritin concentrations as high as 10 mg/ml when analysed by competitive e.l.i.s.a. (results not shown). We coupled this anti-(chicken ferritin) antibody to Sepharose 4B to affinity-purify the chicken ferritin from the partially purified liver homogenate which contained the 22 kDa chicken ferritin band (Fig. 2*a*, lane a). This band is missing from the wash-through fractions from the affinity column (Fig. 2*a*, lane c). The bound and eluted fractions from the affinity column consisted of ferritin particles when viewed in the electron microscope (not shown) and contained a major band at 22 kDa on SDS/PAGE (Fig. 2*a*, lane b).

Although the 22 kDa polypeptide was resolved on SDS/PAGE after affinity chromatography (Fig. 2*b*, lane a), a polypeptide band at 240 kDa was also evident when

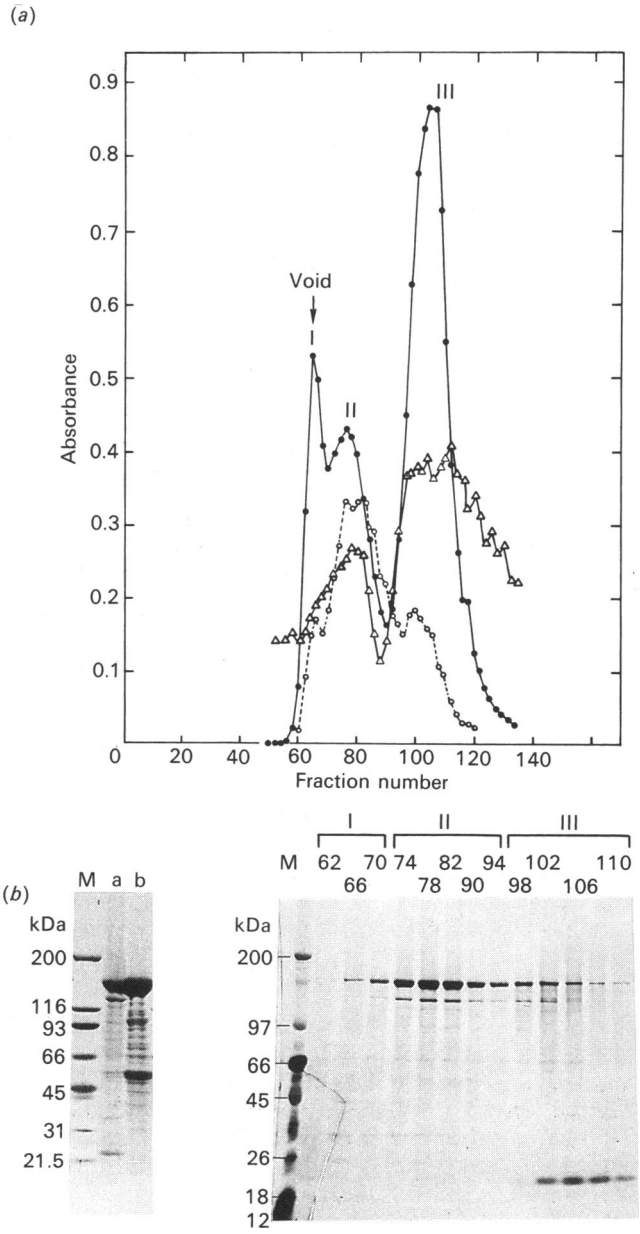

Fig. 1. Purification of chicken liver ferritin by gel permeation chromatography on controlled-pore (1908A) glass beads (*a*) and analysis of the eluted fractions by SDS/PAGE (*b*)

(*a*) A sample (2.5 ml) of partially purified chicken liver homogenate consisting of membrane vesicles and ferritin was applied to a 1.5 cm × 100 cm column of controlled-pore glass beads as described in the Experimental section. Fractions (1 ml) were collected at a flow rate of 6 ml/h and the absorbance was monitored at 280 nm (●). Protein was quanitified by the method of Bradford (1976) (absorbance at 595 nm, ○) and ferritin was assayed by e.l.i.s.a. (absorbance at 490 nm, △). (*b*) Samples for SDS/PAGE (75 μl) were diluted with a 4× concentration of SDS sample buffer (25 μl) and heated at 100 °C for 2 min before 50 μl was applied to a 3.75% to 15% acrylamide gradient gel. Lane a (chicken liver) and lane b (bovine brain) are samples of the homogenates before application to the gel permeation column. Lanes marked I, II, and III contain samples of the numbered fractions from the three column peaks in (*a*) starting with the void peak. M, molecular mass markers.

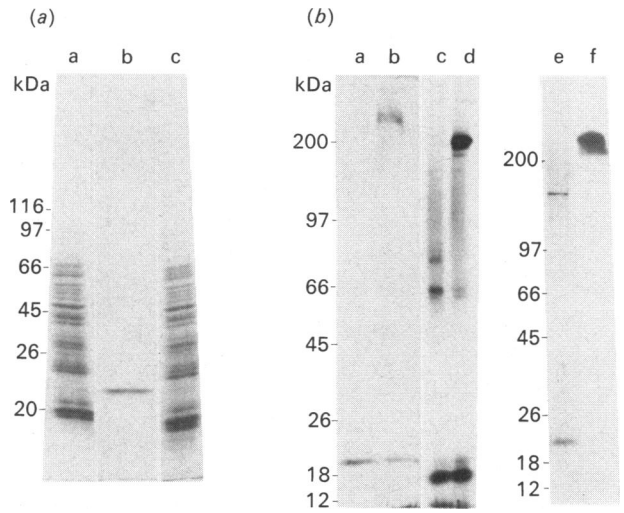

Fig. 2. Purification of chicken liver ferritin by antibody affinity column chromatography and analysis by SDS/PAGE

A sample (10 ml) of 100000 *g*-h supernatant from chicken liver was applied in isolation buffer (IB) at pH 6.5 to a 3.5 ml column of monoclonal antibody coupled to Sepharose 4B at a flow rate of 5 ml/h. The column was then washed with 20 bed vol. of IB. Neither Triton X-100 at 0.1% nor 0.5 M-NaCl eluted protein. However, ferritin was eluted with 0.1 M-triethylamine, pH 11.0, at a flow rate of 10 ml/h. (*a*) Starting material (lane a), triethylamine-eluted antigen (b) and wash-through material (c) were analysed on SDS/PAGE after boiling for 5 min. (*b*) Affinity-purified chicken ferritin (lanes a,b), commercial horse spleen ferritin (c, d), or CPG-column chromatography-purified chicken ferritin (e,f) was incubated at 24 °C (b, d, f) or 100 °C (a, c, e) prior to SDS/PAGE.

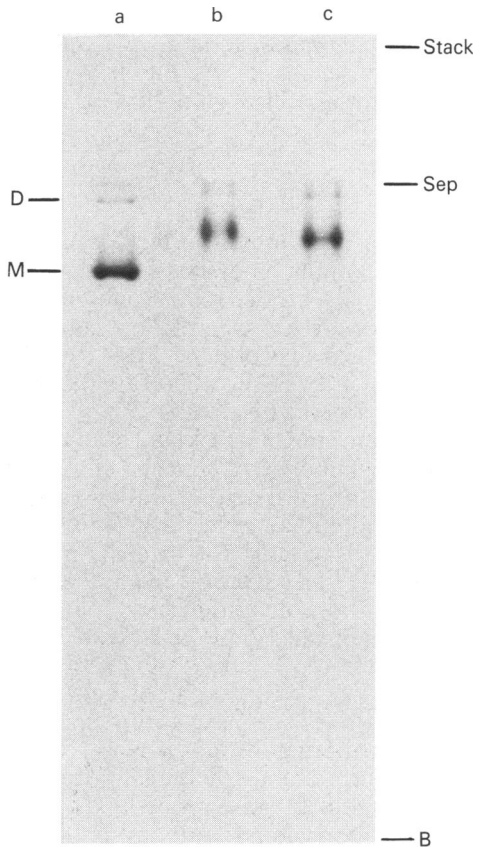

Fig. 3. Non-denaturing PAGE of chicken liver ferritin and horse spleen ferritin

Chicken liver ferritin prepared either by the CPG column method (lane c) or by immunoaffinity chromatography (b), and horse spleen ferritin obtained commercially (a), were electrophoresed on 7% acrylamide slab gels for 16 h at 50 V. Horse spleen monomer (M) and dimer (D) are shown Stack, top of stacking gel; Sep, top of separation gel; B, bottom of gel.

the affinity-purified samples were dissolved in SDS buffer but not heated before electrophoresis (Fig. 2*b*, lane b). Similarly, samples from the included peak III of the CPG column shown in Fig. 1(*a*) contained material at the 240 kDa position when not heated (Fig. 2*b*, lane f), whereas heated samples contained 22 kDa protein (Fig. 2*b*, lane e). A similar pattern was observed with horse spleen ferritin (Fig. 2*b*, lanes c,d). However, the molecular masses of the horse ferritin polypeptides appeared to be 18 kDa, 21 kDa and 210 kDa. The 22 kDa polypeptide of chicken ferritin did not react with anti-(chicken ferritin) antibody after transfer to nitrocellulose, but the 240 kDa protein did bind antibody (results not shown), suggesting that the antibody only recognizes the non-denatured, higher molecular mass forms (240 kDa and intact ferritin) of the molecule.

To determine if chicken and horse ferritin exhibited different overall net charge or size, both molecules were subjected to non-denaturing PAGE (Fig. 3). Intact chicken liver ferritin (lane b) exhibited reduced migration compared to horse spleen ferritin (lane a), suggesting that it was either of a higher molecular mass and/or was less acidic than horse ferritin (see below). CPG-purified chicken ferritin (lane c) appeared to migrate slightly faster than the affinity-purified chicken ferritin on these non-denaturing gels.

Since there appeared to be some difference in the migration of the ferritins on non-denaturing gels, we next wished to determine if there were differences in the isoelectric points of the subunits. Chicken liver and horse spleen ferritins were found to differ when electrophoresed on two-dimensional (isoelectric focusing–SDS/PAGE) gels (Fig. 4). However, as expected, chicken ferritin purified from the gel filtration column was identical to the chicken ferritin purified by antibody affinity chromatography (results not shown). The chicken ferritin molecule resolved into several 22 kDa spots between isoelectric points 6.1 and 6.6 (Fig. 4*b*). The 240 kDa form of the molecule had a pI of 6.6. In contrast, the horse ferritin polypeptides were somewhat more acidic than the chicken ferritin 22 kDa polypeptides, exhibiting isoelectric points ranging from 5.8 to 6.3 (Fig. 4*a*). Unlike chicken ferritin, the higher molecular mass form of horse ferritin was heterogeneous with isoelectric points centred at 5.1 and 4.9. The samples in Fig. 4 consisted of a mixture of heated and unheated ferritin samples in order to resolve both the subunit and the high molecular mass forms of the molecules. When either heated or unheated samples were electrophoresed separately, the isoelectric points of the subunit or the high molecular mass forms of chicken and horse ferritin were the same as in Fig. 4 (results not shown).

Since unheated chicken liver ferritin and horse spleen ferritin have different isoelectric points and different

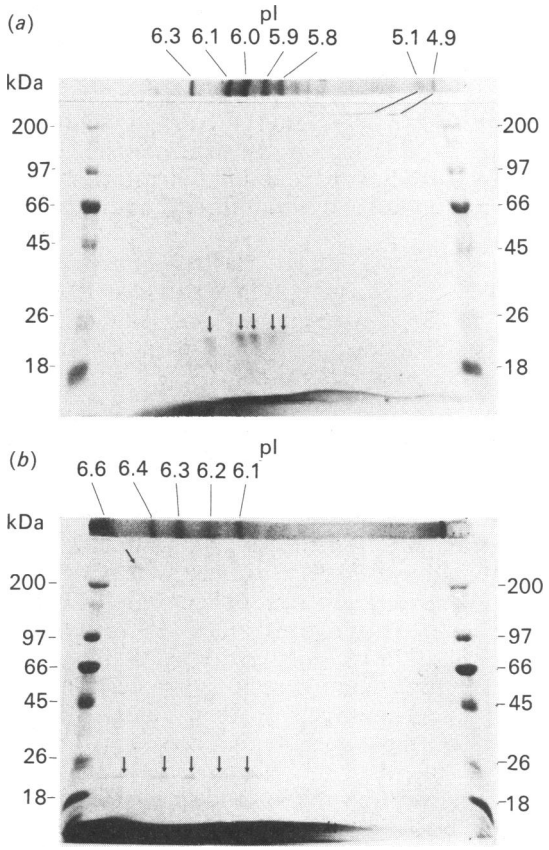

Fig. 4. Isoelectric focusing heterogeneity of horse spleen and chicken liver ferritin on two-dimensional gel electrophoresis.

Horse spleen ferritin (*a*) or chicken liver ferrin (*b*) was prepared for electrophoresis in the first dimension by boiling 86 μg of ferritin in 2% SDS and adding this denatured ferritin to 17 μg of ferritin incubated at 24 °C in 2% SDS in order to reveal the subunit and 240 kDa forms of the molecule. The mixture was electrophoresed in the first dimension (isoelectric focusing), then in the second dimension (3–15% acrylamide slab) and the slab gels stained with silver. Duplicate first dimension gels stained with Coomassie Brilliant Blue are shown above the second dimension gels. Molecular mass standards (M) were applied to both sides of the second dimension gels. Isoelectric points were calculated from a standard curve obtained by measuring the pH of 1 cm sections of duplicate first dimension gels equilibrated in deionized water.

molecular masses on SDS/PAGE, we wished to determine if bivalent cations or thiol groups were important in the maintenance of the chicken ferritin structure. Affinity-purified ferritin was incubated in SDS buffer containing either $CaCl_2$, EGTA, 2-mercaptoethanol, or dithiothreitol before separation on SDS/PAGE. Chicken ferritin treated with $CaCl_2$ or untreated chicken ferritin exhibited a molecular mass of 240 kDa when samples were not boiled, but ferritin incubated with EGTA or 2-mercaptoethanol migrated with a slightly higher molecular mass of 260 kDa (results not shown). Dithiothreitol also induced a molecular mass shift from 240 kDa to 260 kDa in chicken ferritin, but had no effect on the migration of the 210 kDa horse ferritin (results not shown). When the absorption spectrum of chicken ferritin was examined over the range 600 nm–340 nm, it was found to be essentially identical to the spectrum for horse spleen ferritin (results not shown). Neither horse nor chicken ferritins contained distinct absorbance peaks in the visible range of the spectrum.

To compare the diameters of intact chicken liver ferritin and horse spleen ferritin, we negatively stained both for observation in the electron microscope. Each appeared as uniform particles with electron-dense centres (results not shown). The particles had an average diameter of 13.4 ± 0.8 nm for chicken ferritin purified by glass-bead gel permeation chromatography and 12.5 ± 0.9 nm for chicken ferritin purified by affinity chromatography. Horse ferritin particles appeared similar to chicken ferritin with a diameter of 11.0 ± 0.7 nm.

When unlabelled horse and iodinated chicken ferritin were co-chromatographed on Sepharose 4B (results not shown), both were eluted at a position corresponding to a molecular mass of 470 kDa. Similar results were obtained using CPG column-purified chicken ferritin showing that the ferritin used in these studies appeared not to be differentially aggregated or degraded.

DISCUSSION

We have purified chicken liver ferritin by two techniques that have not traditionally been used to purify ferritins and have found that, consistent with the results of other published purification methods, chicken liver ferritin exhibits several unique structural features when compared with horse spleen ferritin. One purification scheme employing gel permeation chromatography resolved ferritin from larger membrane vesicles while immunoaffinity chromatography resulted in the isolation of pure chicken liver ferritin free of essentially all contaminating molecules. The gel permeation method is comparable to other published methods of ferritin purification (Bezkorovainy, 1980). However, since affinity purification is basically a one-step procedure after homogenization of tissue, it is faster and much simpler than previous methods (Crichton *et al.*, 1973).

We next compared the structures of chicken liver ferritin and commercially available horse spleen ferritin in order to further evaluate these purification methods. Isoelectric focusing of pure ferritin showed that chicken liver ferritin subunits consist of a heterogeneous group of peptides of unique apparent molecular mass of 22 kDa, but with isoelectric points of 6.1–6.6. The finding that a unique ferritin subunit molecular mass nevertheless exhibits different isoelectric points on isoelectric focusing gels is consistent with other reports (Drysdale, 1977; Arosio *et al.*, 1978; Otsuka *et al.*, 1981) that showed ferritin sub-bands of the same apparent molecular mass but different charge. In agreement with these reports, we also find that horse ferritin subunits appear to exhibit two molecular masses of 21 kDa and 18 kDa (Fig. 2*a*) on our SDS/polyacrylamide gels. We found by Sepharose 4B chromatography of intact chicken and horse ferritin molecules that both are eluted with an apparent molecular mass of 470 kDa. Therefore, since chicken ferritin exhibits slower migration than horse ferritin on non-denaturing PAGE (Fig. 3), but is of the same apparent molecular mass by sizing chromatography, the intact chicken ferritin molecule is less acidic than the intact horse ferritin molecule. This is consistent with the pI values of 6.14 and 6.27 reported for the intact chicken

spleen ferritin (Shinjyo, 1973) and pI values of 4.34–4.43 (Shinjyo, 1973) and 4.1–4.4 (Stefanini *et al.*, 1982) reported for intact horse spleen ferritin.

Our calculated absorption coefficient for chicken ferritin is 35, which agrees with the value calculated for horse ferritin ($A^{1\%}_{280} = 34$). These high coefficients appear to be due to the high iron content of the ferritins since the $A^{1\%}_{280}$ of horse apoferritin is equal to 9 (Bryce & Crichton, 1973). In agreement with these findings, Yariv *et al.* (1981) found that apo-bacterioferritin exhibits lower absorption than holo-bacterioferritin at 280 nm.

A radioimmunoassay method has been used to quantify chicken ferritin levels using polyclonal antibodies (Calvo *et al.*, 1981, 1982) to measure ferritin levels in serum, liver and spleen. In contrast, we report the first characterization of a monoclonal IgG antibody that recognizes chicken ferritin but does not recognize horse spleen ferritin by competitive e.l.i.s.a. Although ferritins from horse spleen and human liver were found to be related immunochemically (Fine & Harris, 1963), immunoassays have revealed differences in composition between subunits of ferritin from the same species (Otsuka *et al.*, 1981; Stefanini *et al.*, 1982) and between ferritins from different tissues of the same species (Stefanini *et al.*, 1982). Others have found that ferritin from bacteria does not cross-react with antibody to horse spleen ferritin (Yariv *et al.*, 1981). Although much of the structure of ferritin appears to be highly conserved among species, probably due to its universal iron-carrier function, parts of the molecule such as the hinge regions of the helices are exposed on the surface of the molecule and therefore may be more immunogenic than the rest of the molecule (Addison *et al.*, 1984). These surface regions of the molecule bear the epitopes which are responsible for antigenic uniqueness among species and may be recognized by the monoclonal antibody described in this study.

We observed the presence of a 240 kDa chicken ferritin and a 210 kDa horse ferritin intermediate on SDS/PAGE. These proteins are present on SDS/polyacrylamide gels when the samples are not heated. In contrast, after heating, only the 22 kDa subunit of chicken and the 18 kDa and 21 kDa subunits of horse ferritin are observed. It is clear that the 240 kDa form of chicken ferritin is ferritin since monoclonal anti-(chicken ferritin) antibodies bind to this 240 kDa protein after transfer to nitrocellulose. The fact that the horse ferritin 210 kDa protein has a lower apparent molecular mass than the 240 kDa chicken ferritin protein may be due to differences in conformation in SDS, differential SDS binding, or differences in the number of associating subunits.

We took advantage of our ability to resolve the 240 kDa chicken and the 210 kDa horse ferritin proteins on SDS gels to investigate other structural differences between horse spleen ferritin and chicken liver ferritin. We found that treatment with EGTA changes the migration of the 240 kDa protein on SDS gels such that it migrates with an apparent molecular mass of 260 kDa. This suggests that bivalent cations may be involved in the maintenance of a compact ferritin structure, at least in SDS, and that removal of the cations may unfold the molecule such that its migration in SDS gels is retarded. Reducing agents such as 2-mercaptoethanol and dithiothreitol also increase the apparent molecular mass of chicken ferritin to 260 kDa consistent with the unfolding of a compact structure. It is believed that cysteine residues in ferritin become exposed when iron is removed to form apoferritin (Munro & Linder, 1978), suggesting an association of these residues with the iron core of holoferritin. Cysteine has also been shown to release iron from ferritin by acting as a reducing agent (Dognin & Crichton, 1975). In contrast, even though horse spleen ferritin has about three cysteine residues per subunit (Crichton *et al.*, 1973), it does not undergo a dithiothreitol-induced shift in molecular mass.

From negatively-stained electron micrographs of chicken liver and horse spleen ferritins it is clear that chicken ferritin is structurally very similar to horse ferritin. The monomer molecular mass of ferritin from chicken spleen was determined by gel filtration to be 525 kDa, with 21.5 kDa for the subunit molecular mass (Shinjyo, 1973). Ferritins from different human organs have been shown to be heterogeneous in molecular mass (Bezkorovainy, 1980) ranging from 460 kDa in liver and spleen to 550 kDa in heart. Martin-Mateo *et al.* (1983) have reported that ferritin isolated from chicken heart muscle by heating at 70 °C, treatment with pH 4.8 buffers, $(NH_4)_2SO_4$ precipitation and Sephadex G-200 chromatography consisted of a protein of 200 kDa molecular mass by gel filtration and exhibited reduced electrophoretic mobility compared to horse ferritin on non-denaturing acrylamide gels. However, based on our data, it is likely that these authors may have isolated a stable SDS-gel intermediate of chicken ferritin since chicken ferritin from liver (this report) and spleen (Shinjyo, 1973) appear by electron microscopy and gel filtration on Sepharose 4B to consist predominantly of a molecule of 470 kDa and 525 kDa respectively.

Our results indicate that chicken liver ferritin contains a major component the 470 kDa monomer on non-denaturing PAGE, and a minor dimer component similar to chicken spleen ferritin (Shinjyo, 1973) or bovine ferritin (Zampiri & Mason, 1968). Commercially available horse spleen ferritin was found to consist of a faster-migrating form of 470 kDa (Stefanini *et al.*, 1982). A small amount of dimer (940 kDa) was also present. In this report, we have purified chicken liver ferritin by two novel techniques and have shown that chicken liver ferritin is structurally different from horse spleen ferritin. In addition, we have used a very specific antibody which should be useful in investigating several ferritin-related processes: ferritin biosynthesis (Zahringer *et al.*, 1976; Richter & Harrison, 1978), the origin of serum ferritin, the composition of glycosylated ferritin, the receptor-mediated endocytosis of iron, and the mechanism of ferritin degradation.

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