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Purification and Characterization of Recombinant Tadpole H-Chain Ferritin in *Escherichia coli*

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Abstract: The tadpole H-ferritin produced in *E. coli* was purified and its molecular properties were investigated to obtain information about the contribution of the H-subunit in the reaction of iron core formation. All the expressed subunits were assembled into complete holoprotein *in vitro*, presumably 24-mer, and the protein was heat-stable. Electron microscopy revealed that the recombinant ferritin forms spherically and contains iron core. No difference was observed in the absorption spectrum of the expressed protein compared to that of the natural ferritin. The Ouchterlony double diffusion of the expressed protein showed that the H-chain ferritin shares an antigenic determinant with natural tadpole ferritin. Rabbit anti-horse spleen ferritin discriminated the H-ferritin from natural ferritin. The rate of ferritin formation by the recombinant H-chain apoferritin was determined to be higher than that shown by natural tadpole ferritin, which consists of H, M and L-subunits. This phenomenon may be caused by the absence of M and L-subunits in the recombinant H-chain apoferritin.

Key words: characterization, purification, recombinant tadpole H-chain ferritin.

Iron is an essential trace element for almost all living organisms, but its availability is limited by the extremely low solubility of Fe(III). Ferritin is a protein designed to solve such a problem, in an almost spherical form which contains large amounts of iron in a soluble, non-toxic and available form (Theil, 1990; Harrison *et al.*, 1989).

Tadpole ferritin is interesting for studying developmental change of ferritin in circulating red cells. During the larval development of amphibia, red cell iron metabolism changes without affecting hemoglobin synthesis (Theil, 1978), and the circulating red blood cells play an important role in iron storage as indicated by a high concentration of ferritin as well as oxygen transport (Theil, 1980).

In most vertebrates, ferritins are composed of various proportions of genetically distinct two subunit types, H and L-chains (Arosio *et al.*, 1978), having 55% homology in amino acid sequence (Jain *et al.*, 1985), but several crucial differences are present on the outer surface, in the cavity and on the hydrophobic channel sequence (Stefanini *et al.*, 1982; Leibold *et al.*, 1984). Tadpole ferritin consists of three subunit types (H, M and L-chains) instead of two chains H and L, which is strikingly different from other ferritins (Dicky *et al.*, 1987). The structure/function relationship of the subunits is not fully understood.

In order to obtain H-chain ferritin not existing in the tissues, we established a method to produce large amounts (>30%) of tadpole H-chain ferritin in *Escherichia coli* using full-length cDNA for tadpole ferritin H-chain (Kim and Kim, 1994). The expressed H-chain ferritin (H-ferritin) was confirmed by nondenaturing polyacrylamide gel electrophoresis. In this study, the tadpole H-ferritin produced in *E. coli* was purified and its molecular properties were investigated to obtain information about the contribution of the H-subunit in the reaction of iron core formation. This was studied by comparing the iron uptake rate of recombinant H-ferritin to that of natural tadpole ferritin. The structural and immunological properties of the H-ferritin produced in *E. coli* indicate that recombinant H-ferritin was analogous to the natural tadpole blood ferritin, which is in a spherical form and contains an iron core. It also confirmed that the H-chain ferritin is far more active in accumulating iron than natural tadpole ferritin.

Materials and Methods

Materials

The expression plasmid pVUTFH10 and its host *E. coli* JM109 have been described by Kim and Kim (1994). Sodium dodecyl sulfate (SDS), sodium acetate,

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TEMED (N,N,N',N-tetramethylethylenediamine), Tris buffer, Coomassie brilliant blue, ferrous ammonium sulfate and ampicillin were purchased from Sigma Chemical Co. (St. Louis, USA). Bacto-trypton, Bacto-agar and yeast extract were from Difco Laboratory (Detroit, USA). Isopropyl-β-D-thiogalactoside (IPTG), urea and all restriction enzymes used in the experiment were obtained from Promega Biotec (Madison, USA). Acrylamide, N,N-methylene bisacrylamide, ammonium persulfate, agarose and molecular size markers were from Bio-Rad (Hercules, USA) and Sephacryl S-300HR was from Pharmacia (Uppsala, Sweden). Horse spleen ferritin was purchased from Boehringer Mannheim (Mannheim, Germany). Antiserum to horse spleen ferritin was purchased from Sigma. Other chemicals used were of the highest grade commercially available.

Purification of recombinant H-ferritin

E. coli strain JM109 containing the ferritin expression vector pVUTFH10 was grown to an absorbance of 0.4 ~0.7 at 600 nm (Kim and Kim, 1994). Ferritin expression was induced by the addition of 0.1 mM IPTG to the cultures and the cells were incubated for an additional 6 h at 37℃. The purification of recombinant H-ferritin was based on the method of Levi *et al.* (1989) with some modifications. The cells were collected by centrifugation, washed with 20 mM Tris-HCl, pH 7.4, containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), resuspended in the same buffer, and sonicated in an ice bath. The cell debris were removed by centrifugation at 10,000×*g* for 10 min. The supernatant was heated at 75℃ for 10 min, cooled rapidly to 4℃ and centrifuged as above. The heat-treated supernatant was precipitated with 80% saturated ammonium sulfate and applied to ultracentrifugation (90,000 ×*g*, 2 h). The precipitate was resuspended in 20 mM Tris-HCl buffer and purified by gel chromatography using a Sephacryl S-300HR column (16×90 cm). The fractions containing ferritin were collected and concentrated by ultrafiltration over an Amicon PM-30 membrane. The purity of the isolated protein was examined by polyacrylamide gel electrophoresis (PAGE).

Electrophoresis

Purified protein was analyzed by both 12% (w/v) SDS- polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) and 7.5% PAGE in nondenaturing gels (Davis, 1964). Samples for SDS-PAGE were heated at 100℃ for 10 min in a loading buffer of 0.1 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol. These gels were stained for protein using 0.2% Coomassie brilliant blue and for iron using $K_4Fe(CN)_6$ (2%) and HCl (2%) mixed (1 : 1, v/v) immediately before use. To analyze the subunit size of H-ferritin, standard molecular size markers (Bio-Rad) used in SDS-PAGE (12%) were phosphorylase B (Mr, 97,400), serum albumin (Mr, 66,200), ovalbumin (Mr, 45,000), carbonic anhydrase (Mr, 31,000), trypsin anhydrase (Mr, 21,000), and lysozyme (Mr, 14,400).

UV/Vis absorption spectroscopy and electron microscopy

Absorption spectra of natural tadpole ferritin and recombinant tadpole H-ferritin in the ultraviolet/visible region were determined using a Beckmann DU-65 spectrophotometer. The proteins used for the absorption spectroscopy were of the same concentration. Purified H-ferritin was examined under a JOEL JEM 1200 2X-II electron microscope. Protein shells were negatively stained with 5% phosphotungstic acid (pH 6.0) for 5 min. Samples were examined on formvar-coated Cu grids.

Double immunodiffusion

Antiserum to tadpole ferritin was prepared by a series of subcutaneous injections to a New Zealand white rabbit of purified tadpole blood ferritin (200 g) suspended in Freund's adjuvant (50%). The resultant rabbit serum was collected and used directly with appropriate dilutions. Immunodiffusion was performed on 1% agarose gels.

Iron uptake

The kinetics of iron uptake were studied as in a previous report using a molar ratio of iron to protein of 200 : 1 (Chang *et al.*, 1994). Apoferritins were prepared by chemical reduction using 1% thioglycolic acid (pH 5.0) and dialized against 0.1 M sodium bicarbonate (pH 8.0; Kim *et al.*, 1986), followed by 20 mM imidazole buffer (pH 7.0). Protein concentration was determined by the modified Lowry method (Hess *et al.*, 1978). A reaction was initiated by addition of Fe(II) to the apoferritin solution in the Fe/protein ratio of 200. A freshly prepared 1 mM ferrous ammonium sulfate was added to apoferritin (50 μg/ml) in 20 mM imidazole buffer, pH 7.0, resulting in a final concentration of 0.02 mM Fe(II). The formation of the amber iron product was monitored by the increase of optical density at 310 nm. A control experiment was carried out with bovine serum albumin.

Results and Discussion

The tadpole H-ferritin produced in *E. coli* was purified and its properties were investigated in comparison with natural tadpole ferritin. The product of recombi-

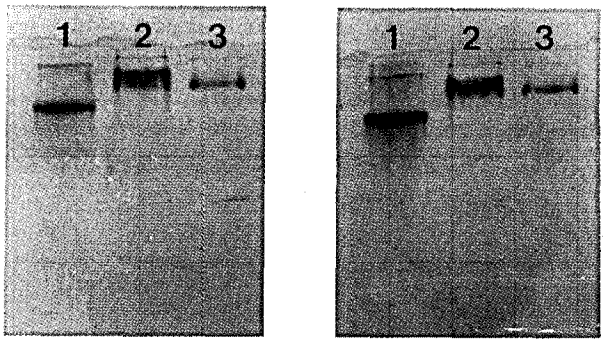


Fig. 1. Nondenaturing electrophoresis (7.5%) of recombinant H-ferritin. Stained with (a) Coomassie blue and (b) ferrocyanide: 1. horse spleen ferritin, 2. recombinant H-ferritin, 3. natural tadpole ferritin.

nant tadpole H-ferritin was purified by gel chromatography on Sephacryl S-300HR. The protein was heat-stable and the heat denaturation step was proven to be effective in removing other proteins. Such heat stability of recombinant ferritin has been reported with ferritin from various sources which include recombinant invertebrate ferritins (Von Darl *et al.*, 1994) and bacterioferritin (Andrews *et al.*, 1993; Hudson *et al.*, 1993) as well as human H- and L-chain ferritins (Levi *et al.*, 1987; 1989). The ferritins engineered in *E. coli* were assembled into spherical proteins as natural ones. The gel chromatography resulted in 2 peaks of which the more attenuated peak included the fractions containing ferritin (data not shown). On PAGE under nondenaturing conditions the H-ferritin showed two closely related bands that stained for both protein and iron (Fig. 1). A slowly migrating faint band appeared over several preparations. This minor faint band was assigned to oligomers of ferritin, as it has been reported to occur in ferritins from other sources (Kim *et al.*, 1986). In both protein and iron stains, it confirms that expressed H-chains assembled into complete holoprotein *in vitro* and have the ability to uptake large amounts of iron into their core. On SDS-PAGE it showed a single band, with a molecular mass estimated to be approximately 23 kDa (Fig. 2). It indicates that recombinant H-ferritin is a homopolymer of the H-chains, presumably 24-mer.

No difference was observed in the UV/Vis absorption spectra between the purified H-ferritin and the natural ferritin (Fig. 3). The spectra contained a broad absorption throughout the visible region, increasing to a shoulder at 280 nm, showing typical ferritin absorption spectra. The broad absorption of recombinant H-ferritin above 310 nm was due to the *in vitro* Fe(II) reaction. There is no spectral evidence for the presence of heme groups.

The protein shell and iron core of the H-ferritin was

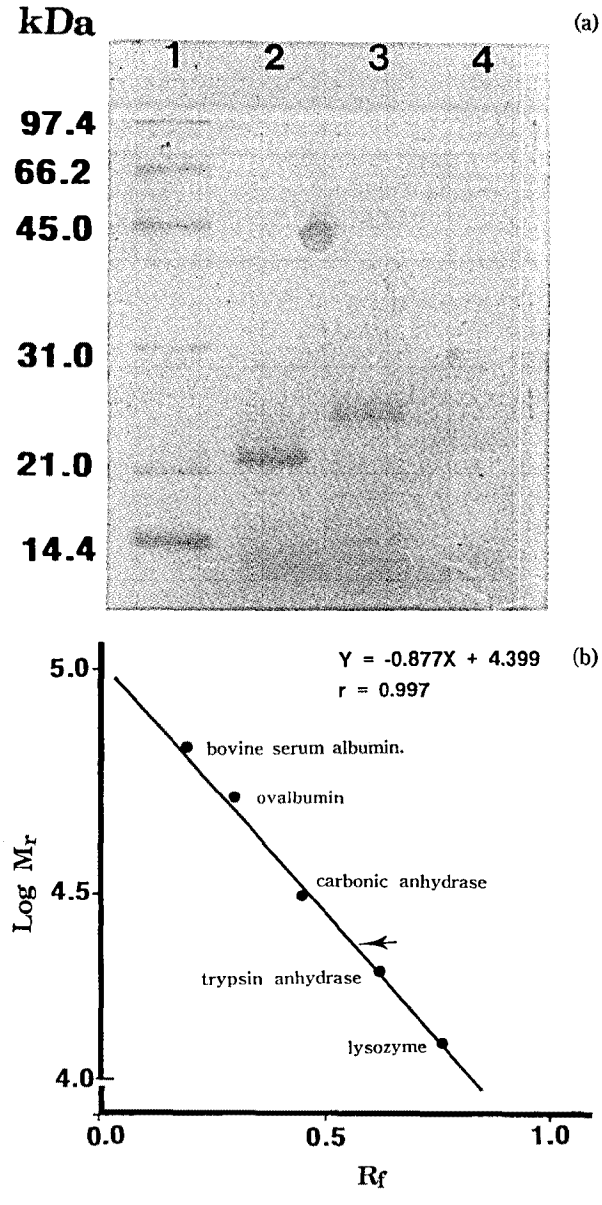


Fig. 2. Molecular weight determination of subunits of the H-ferritin as determined by SDS-PAGE. (a) SDS-PAGE in a 12.5% polyacrylamide gel. 1. size markers, 2. horse spleen ferritin, 3. natural tadpole ferritin, 4. recombinant H-ferritin. (b) Linear regression of the above SDS-PAGE results.

observed under an electron microscope. In negatively stained protein samples the electron dense area represents the ferritin core as clearly shown in the unstained preparation, while the light area surrounding it represents the protein shell (Fig. 4). It proves that the H-ferritin was physically stable and coassembled into a complete spherical form, in which iron core was contained. A remarkable conservation of a three-dimensional structure was analyzed by X-ray crystallographic analyses between naturally occuring ferritins and recombinant ferritins (Harrison *et al.*, 1991). The core size of holo

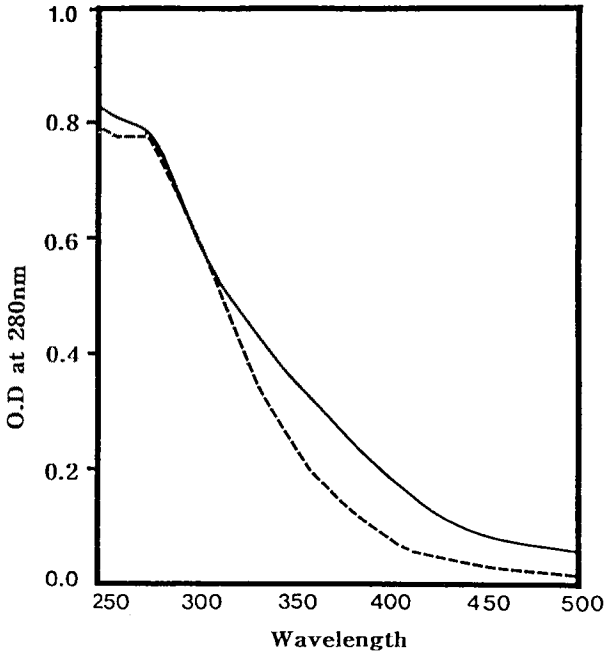
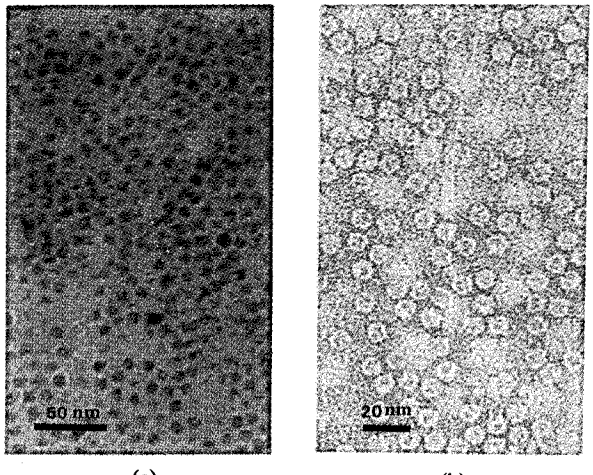


Fig. 3. UV/Vis spectra of recombinant H-ferritin (——) and natural ferritin (-----).



(a) (b)

Fig. 4. Ultrastructure of purified recombinant H-ferritin. (a) unstained ferritin (b) negatively stained with 2% phosphotungstic acid.

protein was estimated to be comparable to the natural tadpole ferritin. A precise size of the core will be reported elsewhere.

On immunodiffusion, the cross-reaction of tadpole ferritin against its antiserum yielded a single precipitin line (Fig. 5a). Recombinant H-ferritin also raised a single precipitin line against the antiserum, which proves that the H-ferritin shares an antigenic determinant with tadpole ferritin. This antiserum showed a faint precipitin line against horse spleen ferritin, although the faint line is not apparent in Fig. 5. A contrary observation was made about immunodiffusion against antiserum to

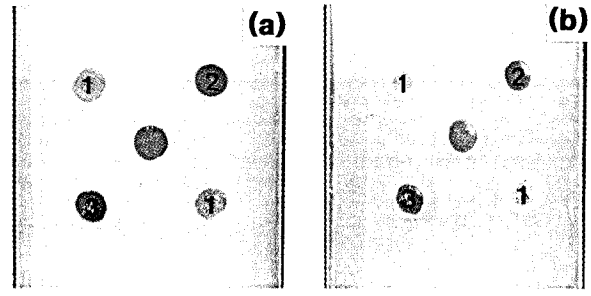


Fig. 5. Comparison of recombinant H-ferritin and natural tadpole ferritin by double immunodiffusion. It was performed against both the antiserum to tadpole ferritin (a) and the antiserum to horse spleen ferritin (b). 1, recombinant H-ferritin; 2, natural tadpole ferritin; and 3, horse spleen ferritin.

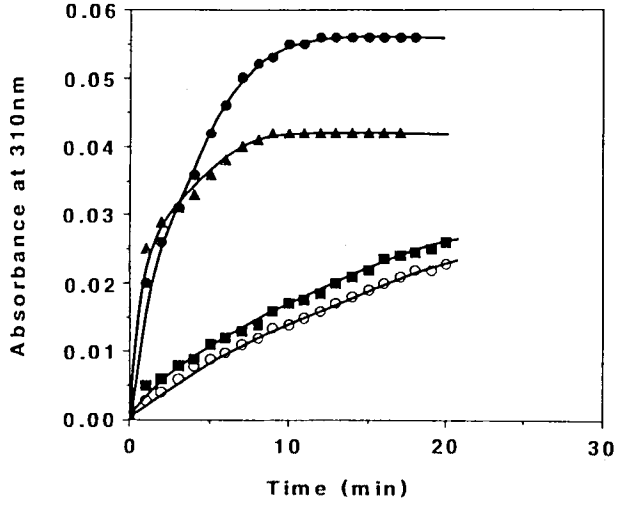


Fig. 6. Iron uptake by recombinant H-ferritin. Protein concentration was 0.1 μM and the ratio of Fe(II) to protein was 200. The buffer used was 20 mM imidazole, pH 7.0, and the amber iron product was monitored at 310 nm. BSA was used as a control. (●–●), Recombinant H-ferritin; (■–■), horse spleen ferritin; (▲–▲), natural ferritin; (○–○), BSA.

horse spleen ferritin (Fig. 5b). This antiserum discriminated the H-ferritin from tadpole ferritin: a single faint precipitin line was seen on the reaction of rabbit anti-horse spleen ferritin against tadpole ferritin whereas no cross-reaction was seen against recombinant H-ferritin. Therefore, antiserum to tadpole ferritin shares antigenic determinants with both the H-ferritin (100% H-chains) and horse spleen ferritin (>90% L-chains). However, antiserum to horse spleen ferritin shares no antigenic determinant with the H-ferritin. A consistant result was obtained from human ferritin H-chain synthesized in *E. coli*. Recombinant human H-ferritin was detected with the monoclonal antibody to human H-chain whereas no precipitin line was obtained with the monoclonal antibody to human L-chain (Cavanna *et al.*, 1983; Levi *et al.*, 1987).

The ability of iron accumulation in the H-ferritin was

examined by using a time scale absorption measurement (Fig. 6). Initial rates and the times needed to reach maximum absorbance, being approximately 10 min, were similar in the reactions but the maximum absorbance was higher in recombinant H-ferritin than the natural ferritin. This difference was even more distinctive against L-rich horse spleen apoferritin and BSA. In earlier studies on tissue ferritins, H-rich ferritins are known to accumulate iron more rapidly than L-rich ferritins (Levi *et al.*, 1989; Santambrosio *et al.*, 1993; Sun *et al.*, 1993). With the aid of recombinant DNA techniques, human H-ferritin produced in *E. coli* showed a higher capacity of iron oxidation than L-ferritin by several-fold (Levi *et al.*, 1989). The uptake rate also increased in proportion to the protein concentration (data not shown). Interestingly, the initial rate by natural tadpole ferritin was apparently higher than that by recombinant H-ferritin. The reason is not clear but may be related to the L-chains present in natural ferritin. In the case of human ferritin, the L-chain is known to have a role in protein stabilization and iron mineralization (Levi *et al.*, 1992; 1994; Sun *et al.*, 1993).

Taken together, present data confirms that the H-ferritins produced in *E. coli* readily folded and assembled into holoprotein *in vitro* to be analogous to the natural ferritin. The H-ferritin appears to be far more active in accumulating iron than natural ferritin which consists of H, M and L-subunits. The determination of the initial rate and kinetic parameters of iron deposition is yet to be made. Further mutational analyses of our recombinant H-ferritin will confirm a site of ferroxidase in this protein so far known only to human H-ferritin.

Acknowledgement

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