Biol Metals (1990) 3:222-226

BIOLOGY OF METALS



# β-Thalassaemia/haemoglobin E tissue ferritins

## I: Purification and partial characterization of liver and spleen ferritins

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Received August 16, 1990

**Summary.** Ferritins from liver and spleen of both β-thalassaemia/haemoglobin E (HbE) and non-thalassaemic patients were purified by heating a methanol-treated homogenate, followed by molecular exclusion chromatography. The concentrations of ferritins in the β-thalassaemia/HbE liver and spleen were calculated as 3.8 and 2.0 mg/g wet tissue. The β-thalassaemia/HbE ferritin iron/protein ratios were higher than those of normal ferritins. On PAGE, all ferritins gave a single major monomeric band with only very small differences in their mobility. Ferritins from thalassaemic patients also possessed bands corresponding to oligomers. On SDS/PAGE, all ferritins were resolved into two major subunits: H and L with L subunit predominating. While the isoferritin profiles of ferritins from β-thalassaemia/HbE liver and spleen were similar to each other and to those of normal liver and spleen, some extra bands were present in the acidic region. The microstructure of these pathological ferritins appears to result, to a large degree, from the particular nature and amount of iron loading present.

**Key words:** Ferritin - β-Thalassaemia - Haemoglobin E - Liver - Spleen

### Introduction

β-Thalassaemia is a common genetic disorder of haemoglobin synthesis occurring throughout the world. While it is usually thought of as originating in Mediterranean countries, the gene frequency in Thailand and Laos can reach up to 9% (Wasi 1981). In addition, a haemoglobinopathy, haemoglobin E, also occurs at very high gene frequencies (up to 50%) in south-east Asia leading to a large number of patients with both β-thalassaemia and haemoglobin E (Wasi 1981). In contrast to the situation in most countries around the Mediterranean, the Thai β-thalassaemia/haemoglobin E population receives little or no medical treatment in the form of blood transfusions. However, despite the lack of treatment, the Thai β-thalassaemia/HbE population has been shown to be heavily iron-loaded (Wasi 1981), a situation arising through an increased iron absorption from their diet (Pootrakul et al. 1988). Much of this excess iron occurs in the form of ferritin and haemosiderin stored within the tissues (Bhamarapravati et al. 1967).

Ferritin is a well known mammalian iron-storage protein, composed of a multisubunit protein shell (apoferritin) surrounding an iron core which contains up to 4500 iron atoms, the amount depending on the flux of iron into the body (Harrison et al. 1980). The structure of ferritin also varies in terms of subunit composition, isoferritin profile, as well as the size and crystallinity of the biomineral core, depending on the particular organs analysed and pathological condition of the patient (Powell et al. 1975; Bomford et al. 1978; Mann et al. 1986; St. Pierre et al. 1989). The predominant form of iron in iron-overload conditions is haemosiderin (Peters et al. 1977) which also varies in structure with the pathological condition of the patient. Thus, the core structure of haemosiderin isolated from idiopathic haemochromatosis patients differs significantly from that isolated from patients who have received repeated blood transfusions in order to counter the chronic anaemia associated with thalassaemia (Mann et al. 1988). In addition, the mode of formation of haemosiderin is still a subject for controversy, with some authors suggesting it is formed from the degradation of ferritin, while others claim it is formed independently (Andrews et al. 1987; Mann et al. 1988).

The marked heterogenety of both ferritin and haemosiderin isolated from various iron-overload pathologies suggests that differences may be found between these proteins obtained from patients suffering from β-thalassaemia/HbE and those from patients who have received repeated blood transfusions. While many pre-

*Abbreviations.* PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate

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vious studies have characterized ferritin from multitransfusional homozygous β-thalassaemia patients (see e.g. Mann et al. 1986), virtually no work has been carried out on characterizing ferritins from β-thalassaemia/HbE patients. This study is thus aimed at elucidating the structure of liver and spleen ferritin from non-transfused Thai β-thalassaemia/HbE patients. The liver and spleen have been selected as they play a primary role in iron metabolism. This paper forms the first of a series of studies on the iron-storage proteins isolated from such patients. In the following paper (Tran et al. 1990) ferritins from heart and pancreas tissues are compared with those of liver and spleen.

## Materials and methods

*Isolation of ferritin.* Samples of liver and spleen were obtained from β-thalassaemia/HbE patients and were supplied by the Thalassaemia Centre, Siriraj Hospital, Bangkok, Thailand. For comparison, samples of human liver and spleen were obtained from non-thalassaemic patients at Queen Elizabeth II Hospital, Perth, Western Australia, while horse spleen was obtained from Murdoch University.

The purification procedure used was a modification of that of Cham et al. (1985) which involves heating a methanol-treated homogenate. Briefly the method is as follows. After removal of fatty tissue by dissection, samples from all three sources were homogenized with ice-cold 0.05 M phosphate-buffered 0.15 M saline in the volume ratio of 1:4. Phenylmethylsulphonyl fluoride (0.12 mM) was added as a protease inhibitor, and the mixture was centrifuged at 2000 *g* (max) for 15 min. Methanol was added to the supernatant in a volume ratio of 2:5 and the mixture heated to 75° C for 10 min. After cooling in ice, samples were again centrifuged at 2000 *g* (max) for 15 min and then the clear brown supernatant concentrated by ultrafiltration through an Amicon PM30 membrane. This concentrated solution was further purified by molecular exclusion chromatography using a column (2.6 × 40 cm) of Sephadex G-75 and, after reconcentration over an Amicon PM30 membrane, a column (2.6 × 120 cm) of Sephacryl S300. The fractions containing ferritin were then concentrated by ultracentrifugation (110000 *g* max, 1 h). The final product was judged to be pure due to its staining for both protein and iron on PAGE. Ferritin purified in this manner was stored in 25 mM $Na_2B_4O_7$ buffer pH 8.6 containing 0.1% (mass/vol.) $NaN_3$ to prevent bacterial growth.

*Characterization methods.* Protein concentrations were determined by the method of Hess et al. (1978) using bovine serum albumin as a standard. Iron concentrations were measured using a ferrozine iron assay (Kaldor 1958; Stookey 1970; Yee and Goodwin 1974). Quantitative determination of ferritin was carried out using two-site enzyme immunoassay (Flowers et al. 1986).

PAGE in 5% gels was performed using a Tris/glycine non-dissociating discontinuous buffer system (pH 8.8) at 10° C with a current of 10 mA for the first 10 min and 50 mA for the remaining time. Gels were stained for protein using Coomassie brilliant blue R-250 and for iron using $K_4Fe(CN)_6$ (2%), and HCl (2%), mixed 1:1 (by vol.) immediately before use (Perls reagent).

Subunit masses were determined by dissociation of ferritin by heat treatment (60° C, 15 min) in the presence of SDS (3%) and 2-mercaptoethanol (3%) followed by PAGE on 15% SDS gels (Laemmli 1970; Hames 1981). Molecular mass markers (Sigma) used were lysozyme ($M_r$ 14300), β-lactoglobulin (18400), trypsinogen (24000), pepsin (34700), egg albumin (45000) and bovine serum albumin (66000). The $M_r$ values were calculated from the linear regression curve of log $M_r$ of the standards versus the relative mobility of the protein bands.

Isoelectric focussing was carried out over the pH range 4.0–6.5 using polyacrylamide gel plates (5%) containing carrier ampholytes (LKB). Electrophoresis was performed at 10° C using 2000 V, 25 mA and 25 W for 2.5 h. Protein and iron staining was conducted as described above.

## Results

Following the purification, the amount of ferritin isolated from approximately 100 g β-thalassaemia/HbE liver was approximately 80 mg. Immunoassay showed that the concentration of ferritin present in the original tissue homogenate was 0.88 mg/ml and thus the yield was 20% (Table 1). Using the assumption that all the tissue ferritin was extracted into the supernatant during the homogenization step, the concentrations of ferritin in the liver and spleen can be calculated as 3.8 and 2.0 mg/g wet tissue, respectively. The iron/protein ratios for the several ferritins isolated ranged from a minimum of 0.16 for a single preparation of non-thalassaemic liver ferritin and 0.18 for non-thalassaemic spleen to 0.22 and 0.23 for β-thalassaemia/HbE liver and spleen ferritins, respectively.

Following PAGE and protein staining, a single major band was present for ferritins isolated from both β-thalassaemia/HbE and non-thalassaemic liver and spleen (Fig. 1). However, the non-thalassaemic spleen band migrated slightly faster than the other samples (Fig. 1). Two minor bands were present in both β-thalassaemia/HbE liver and spleen, presumably corresponding to oligomers of ferritin. An identical profile was obtained after iron staining as was found following staining for protein.

SDS/PAGE of liver and spleen ferritins from β-thalassaemia/HbE tissue, non-thalassaemic tissue and horse spleen gave two prominant bands in all cases, indicating the presence of two different subunits (Fig. 2). The bands were of unequal intensity with the light (L) subunit showing by far the heavier staining in all cases. In all tissues the light subunit corresponded to 19 ± 0.5 kDa while the $M_r$ of the heavy (H) or slower migrating band from human tissues corresponded to 22.5 ± 1 kDa. The $M_r$ of the heavy subunit from horse spleen ferritin corresponded to 21 ± 0.5 kDa. In addition to the two major bands, several minor bands were

**Table 1.** Ferritin yields during purification

| Stages | Volume (ml) | Ferritin concentration (mg $ml^{-1}$) | Yield (%) |
|---|---|---|---|
| 1. Supernatant after homogenizing and centrifugation | 450 | 0.88[a] | 100 |
| 2. Supernatant after $CH_3OH$ and heat treatment | 450 | 0.53[a] | 60 |
| 3. Purified ferritin at end of procedure | 22 | 3.6 | 20 |

[a] Two-site enzyme immunoassay

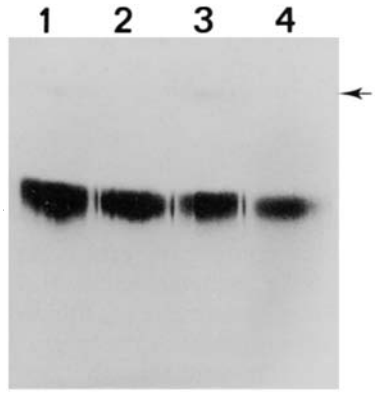


**Fig. 1.** Polyacrylamide gel electrophoresis of ferritins isolated from (1) $\beta$-thalassaemia/HbE liver, (2) non-thalassaemic liver, (3) $\beta$-thalassaemia/HbE spleen and (4) non-thalassaemic spleen. In this and all subsequent figures the gels were stained for protein using Coomassie brilliant blue R-250. *Arrow* denotes the position of the oligomeric ferritin band found in $\beta$-thalassaemia/HbE tissues

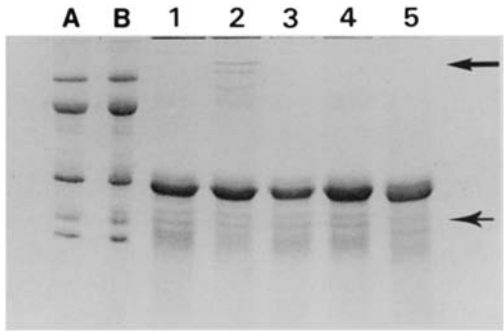


**Fig. 2.** SDS/PAGE, on 15% (mass/vol.) acrylamide gel of ferritins isolated from (1) non-thalassaemic spleen, (2) $\beta$-thalassaemia/HbE spleen, (3) non-thalassaemic liver, (4) $\beta$-thalassaemia/HbE liver and (5) horse spleen. Molecular mass standards are shown in tracks A and B. Note the presence of low-molecular-mass components in all samples (fine arrow) and high-molecular-mass components in $\beta$-thalassaemia/HbE spleen ferritin (*broad arrow*)

observed at staining intensities that were relatively very low. $M_r$ for the minor bands were determined to be 61.7, 50, 17 and 15.5 kDa and are assigned, variously, to oligomers of subunits and partially degraded subunits of ferritin (Fig. 2).

On isoelectric focussing, ferritins from both $\beta$-thalassaemia/HbE and non-thalassaemic liver and spleen showed a complex pattern of bands that were appreciably more basic than those of horse spleen ferritin (Fig. 3). Thus, liver and spleen $\beta$-thalassaemia/HbE ferritin gave a range of pI values over 4.8–5.7. Prominant bands were found in liver and spleen ferritin from both $\beta$-thalassaemia/HbE and non-thalassaemic patients at pI of approximately 5.6. However, while the most basic ferritin found from both organs was the same as that from $\beta$-thalassaemia/HbE material (5.5–5.6), both liver and spleen lacked the very acidic ferritins found in the $\beta$-thalassaemia/HbE organs and gave a lower pI of only 5.0 and 4.9, respectively (Fig. 3). In contrast, horse

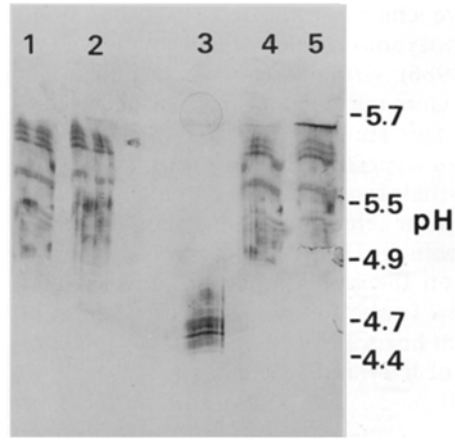


**Fig. 3.** Isoelectric focussing profile over the pH range 4.0–6.5 of ferritins isolated from (1) $\beta$-thalassaemia/HbE liver, (2) $\beta$-thalassaemia/HbE spleen, (3) horse spleen, (4) non-thalassaemic spleen and (5) non-thalassaemic liver

spleen ferritin was far more acidic, showing a pI range of 4.4–4.8 (Fig. 3). On staining for iron, the pI gel gave an almost identical pattern to that found after protein staining.

## Discussion

This study has shown that relatively large amounts of ferritin are found in the livers and spleens of non-transfused $\beta$-thalassaemia/HbE patients. It has also shown that, following extraction and purification, this ferritin is in many ways similar to that from non-thalassaemic sources, but some differences can be detected.

The purification procedure, which is based on the solubility of ferritin in 40% methanol and its heat stability at 75° C, was extended from that of Cham et al. (1985) due to the presence of contaminating material remaining after the heating step. The inclusion of molecular exclusion chromatography and associated ultrafiltration reduced the overall yield of ferritin from 60% to 20%, which is lower than the 40% yield reported in the original method (Cham et al. 1985). However, the modified procedure resulted in a completely pure preparation. This technique is simpler than the multiple-step purification of ferritin normally used which includes heating, changing pH, ammonium sulphate precipitation, resolubilization and gel chromatography (see Harrison et al. 1980).

The ferritin concentrations of $\beta$-thalassaemia/HbE liver and spleen (3.8 and 2.0 mg/g wet tissue, respectively) can be compared with those of Nishi (1985) who reported ferritin levels of 0.98 and 1.04 mg/g wet tissue for non-thalassaemic liver and spleen, respectively. The increase in ferritin in both organs is in agreement with the data of Shuler et al. (1990) who showed that the total tissue iron increase in $\beta$-thalassaemia/HbE patients when compared to normal individuals was of the order of tenfold and threefold for the liver and spleen,

respectively. The increase in iron is also reflected in the ferritin iron/protein ratio which rose from 0.16 (normal) to 0.22 in the thalassaemic liver and from 0.18 to 0.23 in the normal and thalassaemic spleen, respectively.

The detailed 'micro structure' of ferritin seems to depend, to a large degree, on the nature of the iron loading. In the normal situation, isoferritin profiles are organ-specific, with that of the liver being slightly less acidic than that of the spleen, while those of the heart, pancreas and kidney are much more acidic when compared to liver and spleen (Powell et al. 1974; Drysdale et al. 1977). Ferritins obtained from patients suffering from alcoholic cirrhosis and transfusional iron overload show the normal organ distribution in isoferritin profile. In contrast, ferritins isolated from heart, kidney, pancreas and spleen of patients with untreated idiopathic haemochromatosis are all remarkably uniform and closely resembled those isolated from the liver of both haemochromatosis and normal patients (Powell et al. 1974).

In many ways iron loading through $\beta$-thalassaemia/HbE is similar to that found in idiopathic haemochromatosis in that in untreated patients the rate of iron absorption from the gut is elevated throughout life. This is in contrast to the more rapid iron loading which occurs following repeated blood transfusions. This is reflected in the results obtained, namely, that close similarities were observed on PAGE, SDS/PAGE and isoelectric focussing between ferritins from $\beta$-thalassaemia/HbE liver and spleen and ferritins obtained from non-thalassaemic liver.

The presence of oligomeric bands on PAGE from ferritins from $\beta$-thalassaemia/HbE liver and spleen parallels the finding of Powell et al. (1975) who described the presence of these bands in some cases for ferritins isolated from normal sources. Their presence in both organs in all individuals in our study implies that some form of conformational change has occurred in the protein shell leading to a greater degree of polymerization. However, the nature of this change is not known. No evidence was found for the 'light' ferritin bands described as being present in siderotic mice (Massover 1985).

The domination of subunit composition by the L chain agrees well with the literature where it has been established that the L subunit is the major form manufactured during long-term iron storage (Bomford et al. 1981). No difference in subunit mass was found between ferritins of non-thalassaemic and $\beta$-thalassaemia/HbE origin. However, it is of interest that the heavy subunit gave an $M_r$ of 22500 which is somewhat larger than the 21000 found for horse spleen ferritin.

The presence of minor bands on SDS/PAGE could be the result of inter-subunit disulfide bonding between individual subunits or of changes in the intrachain disulfide conformation of subunits (Arosio et al. 1978) or of proteolytic processes (Lavoie et al. 1977). In addition, the multigene nature of ferritin has been well established (Costanzo et al. 1984; Theil 1987) and these bands could well represent some minor gene products.

The heterogeneity of ferritin is not well understood and might derive from apoferritin shells of differing primary structures (Powell et al. 1975). The ferritin molecule has 24 subunits, composed of two polypeptides, H and L. Accordingly, the molecule may contain many isomers depending on the relative proportion of each subunit in the molecule and this could be a cause of the heterogenity of isoferritin profiles (Nishi 1985; Theil 1987). The existence of a multigene family coding for ferritin could also explain the presence of additional bands on the isoelectric focussing profile when compared to that of normal patients.

While few differences were found in the gross structure of the protein shell between ferritin isolated from the liver and spleen from patients suffering from iron overload due to $\beta$-thalassaemia/HbE and ferritin from these organs from patients with normal iron levels, it does not preclude differences being present in the iron cores. Such investigations of the molecular pathology of these biomineral deposits can be conducted via Mössbauer spectroscopy and electron microscopy and will be the subject of future publications.

*Acknowledgements.* This work is supported by the award of a Commonwealth Research Scholarship to K. C. Tran, by the Australian Research Council and by the Murdoch University Special Research Grant Scheme. We thank Dr. Prapon Wilairat and the Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand for providing facilities to isolate and purify ferritin. In additon, P. P. acknowledges the support provided by the United States Public Health Research grant HL34408 from the National Heart, Lung and Blood Institute, USA.

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