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Note

Separation by high-performance liquid chromatography of two types of subunit from horse spleen ferritin

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Understanding the mechanism by which ferritin stores and mobilizes iron depends upon a thorough understanding of the chemical nature and structural organization of the protein subunits of ferritin. Currently, a major problem which must be solved before the ultimate questions about structure/function can be fully answered is the problem of how many chemically distinct subunits may exist in the 24 equivalent positions[1] of the ferritin quaternary structure. It is generally accepted that ferritins from different tissues can be distinguished electrophoretically. In addition, isoelectric focusing has shown heterogeneity within, as well as among, tissue ferritins and suggests that many tissue ferritins contain common "isoferritins" (reviewed in ref. 2). Based on their observation by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) that two different-sized subunits, designated "H" and "L", existed in different ratios in ferritins from various tissues, Drysdale and coworkers[3,4] suggested that multiple isoferritins represented hybrid molecules composed of different proportions of the two subunit types. The ultimate test of this model will be the separation of two or more different types of ferritin subunit in sufficient amount and purity to allow sequence determination of each.

The recent advances made in the use of high-performance liquid chromatography (HPLC) for protein separation[5] prompted this investigation which represents the first use of HPLC for separating the different types of ferritin subunit. The procedure described affords complete purification of each ferritin subunit type in amounts sufficient for subsequent sequence analysis.

EXPERIMENTAL

Horse spleen ferritin was obtained from three sources: a twice-crystallized preparation from Miles Labs. (lot 63A), lot No. 1089305 from Boehringer-Mannheim, and a sample prepared in our laboratory according to the procedure of May and Fish[6] from fresh horse spleens.

Ferritin subunits were routinely prepared by performing simultaneous iron reduction and subunit dissociation via treatment of the ferritin with acetic acid–thioglycolic acid and subsequent gel chromatographic exchange into 0.01 *M* glycine, pH 2.85, or 0.1% trifluoroacetic acid (Collawn and Fish, unpublished results). Subunits prepared in this manner behaved by analytical ultracentrifugation and circular di-

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chroism in a similar fashion to subunits prepared from apoferritin by the method of Harrison and Gregory[7].

HPLC was performed on a Varian 5060 liquid chromatograph. A C_{18} reversed-phase column of 300-Å pore size (SynChropak RP-P, 25 cm × 4.1 mm I.D., SynChrom, Linden, IN, U.S.A.) was employed for the protein separation. The column was maintained at a constant temperature of 30°C, and elution was conducted at a solvent flow-rate of 1 ml/min. The solvent systems employed were: solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile. Although a number of different solvent programs resolved the two subunit species, the following program was used for routine preparation of the two subunits: injection of the sample at 100% A, a linear gradient from 0% B to 75% B over 25 min, and an isocratic elution at 75% B for 3 min.

SDS-PAGE was performed on 1.5 mm × 12 cm × 18 cm gels of 15% polyacrylamide in a Bio-Rad apparatus. The discontinuous buffer system of Laemmli[8] was employed. Isoelectric focusing was on pH 3–10 slab gels according to the modifictions of Ames and Nikaido[9] and Lavoie *et al.*[10] for the O'Farrell procedure[11].

RESULTS

As illustrated by the elution profile shown in Fig. 1, profile a, two chromatographic species were observed when subunits from commercial horse spleen ferritin were subjected to reversed-phase HPLC. When each of the chromatographic species was re-chromatographed using the same solvent-gradient program, it eluted as a single peak at the same binary solvent composition as in its initial elution (*cf.* Fig. 1, profiles b and c). Estimates of the relative proportions of each of the chromato-

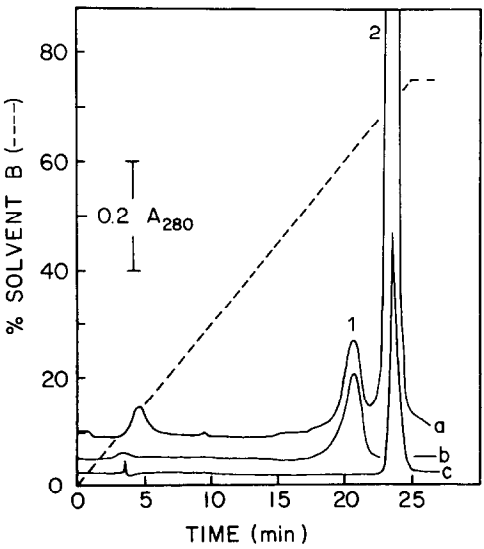

Fig. 1. Reversed-phase HPLC of horse spleen ferritin subunits. Profile a, horse spleen ferritin subunits. Profile b, re-chromatography of peak 1. Profile c, re-chromatography of peak 2. (- - - -) solvent gradient expressed as %B.

graphic species and of the recovery of protein from each column run indicated that 0.08 mg of protein from peak 1 and 0.69 mg from peak 2 were recovered from each milligram of protein applied to the column. This amounted to a 77% overall recovery of protein from each run. A number of ancillary experiments indicated that most of the protein which remained adsorbed to the C_{18} column was species 2; this protein could be cleared from the column by a wash cycle in which the column was repeatedly washed with gradients of 0–75% solvent B. The same two chromatographic species were obtained when a second source of commercial ferritin (purified by a different procedure) was used. Similarly, subunits from horse spleen ferritin which was prepared from fresh tissue without crystallization[6] yielded the same two HPLC species in the same proportions as observed for the commercial ferritins.

The same two chromatographic species were obtained when horse spleen ferritin was dissociated and the iron reduced by treatment of ferritin in 6 *M* guanidinium chloride plus 1% thioglycolic acid, pH 2.85, before subjection to HPLC. Likewise, subunits which were reduced and carboxymethylated in 6 *M* GdmCl, either prior to HPLC separation or after subjection to gel chromatography in 6 *M* GdmCl, eluted

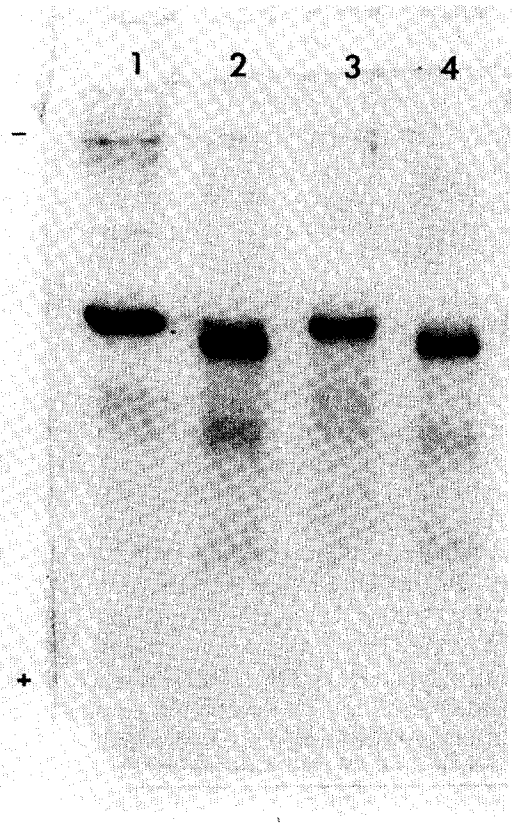

Fig. 2. SDS-PAGE electrophoresis of ferritin subunits separated by HPLC. Samples: 1 = porcine spleen ferritin subunits[6] included for reference; 2 = horse spleen ferritin subunits before chromatography; 3 = HPLC peak 1; 4 = HPLC peak 2.

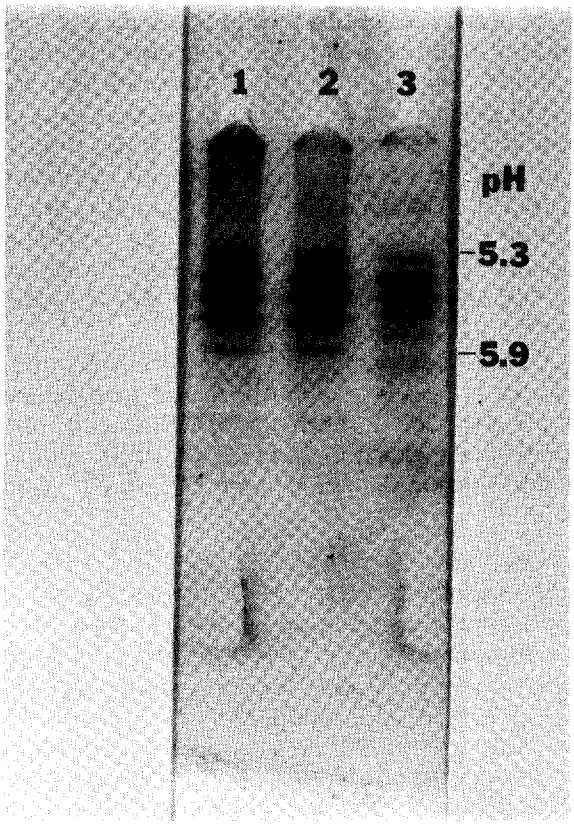

Fig. 3. Isoelectric focusing of ferritin subunits separated by HPLC. Samples: 1 = HPLC peak 2; 2 = horse spleen ferritin subunits before chromatography; 3 = HPLC peak 1.

at their characteristic binary solvent composition upon HPLC. When 2-propanol was employed instead of acetonitrile as the less polar solvent, the same two polypeptides (as identified by SDS-PAGE) were obtained in approximately the same ratio. The preceding three observations argue against one of the two HPLC species being an artifact of the subunit preparation or the elution procedure.

Fig. 2 demonstrates that the two chromatographic species could be differentiated by SDS-PAGE. All other things being equal, the relative electrophoretic mobilities of the two polypeptides suggest that species 1 is slightly larger than species 2 by a molecular weight difference of *ca.* 2000. This was substantiated by gel chromatography of the two polypeptides on Sephacryl S-200 in the presence of 6 *M* GdmCl. By labeling one of the polypeptides with iodo[^{3}H]acetate, the reduced, carboxymethylated derivatives of both species could be compared simultaneously. When so compared, species 1 eluted slightly ahead of species 2. The elution difference corresponded to an apparent molecular weight difference of *ca.* 2000. Since gel chromatography in random-coil-producing solvents is generally less fraught with anomalies than is SDS-PAGE[12], this observation adds considerable credence to the postulated size difference between the two polypeptides first observed by SDS-PAGE[13].

As illustrated in Fig. 3, differences could also be seen between the isoelectric focusing (IEF) patterns of the two polypeptides. Consistent with previous reports for unfractionated horse spleen ferritin subunits[13,14], we observed multiple bands which

TABLE I

AMINO ACID COMPOSITIONS OF HPLC FRACTIONS*

Amino acid	*HPLC No. 1*	*"H" subunit***	*HPLC No. 2*	*"L" subunit***	*Sequence****
Asx	27	19	18	16	18
Thr	7	7	6	5	6
Ser	10	9	10	8	10
Glx	29	23	23	22	26
Pro	4	–	2	–	2
Gly	10	12	12	9	11
Ala	14	15	16	14	15
Val	6	8	9	7	8
Met	3	3	3	3	3
Ile	6	6	4	3	4
Leu	22	20	28	25	28
Tyr	6	6	6	6	6
Phe	8	7	8	7	8
Lys	13	11	9	9	9
His	9	6	6	4	6
Arg	7	9	12	9	11

* Trp and ½Cys were not quantitated.
** Ref. 13.
*** Ref. 5.

focused between pH 5.0 and pH 5.9. Though each species from the HPLC separation exhibited multiple IEF components, those components of HPLC species 1 as a whole appeared slightly more acidic than those of HPLC species 2. Multiple components were also observed for horse ferritin "H" and "L" subunits[13].

Finally, Table I presents the amino acid compositions of HPLC species 1 and 2. Also included for comparison are the amino acid compositions of "H" and "L" subunits from horse ferritin[13] as well as the composition of horse spleen ferritin based on its amino acid sequence[15]. Two points are worth mentioning in regard to a comparison of these data. First, with the exception of Asx, Leu, Pro, and the basic amino acids, the compositions of HPLC species 1 and 2 are indistinguishable. Second, the amino acid composition of HPLC species 1 more closely resembles that of "H" subunit while the amino acid composition of HPLC species 2 is more like those published for "L" subunit and the amino acid sequence for horse spleen ferritin subunit.

DISCUSSION

The major point to be defended in this report is whether the minor component isolated by HPLC is truly an apoferritin subunit and not just a minor contaminant which co-purifies with ferritin. Though the evidence presented herein cannot resolve this question unequivocally, it and a substantial amount of information from other laboratories strongly support our contention that this polypeptide is indeed a ferritin subunit. First, HPLC-species 1 is found in horse spleen ferritin preparations which have been purified in three different ways. These include separations by size, charge, and repeated crystallization. It is highly unlikely that the same protein would survive so many different types of discriminatory procedures always to appear in the same ratio with the major apoferritin subunit species. Second, the chemical and physical properties of HPLC species 1 are quite similar to those of the major component,

HPLC species 2, which suggests that the two are closely related. Third, there is, of course, evidence from other laboratories that horse spleen ferritin has two types of subunit which can be differentiated by SDS-PAGE[13]. Additionally, earlier investigations led by Drysdale's and Listowsky's laboratories have shown that the ratios of the two peptide species vary from one type of tissue to the next[10,13]. In fact, ferritin "H" and "L" subunit types have been observed in various ratios from different tissues for a variety of animal species (reviewed in ref. 2). In this regard, we have recently observed that porcine ferritin subunits can also be separated into more than one chemically distinct species by reversed-phase HPLC[16].

If one accepts the premise that both HPLC fractions are indeed ferritin subunits, it then becomes tempting to speculate that HPLC species 1 corresponds to the "H" subunit and HPLC species 2 corresponds to the "L" subunit. This correlation is based on their relative behaviors on SDS-PAGE (Fig. 2), on their behaviors during gel chromatography in 6 *M* GdmCl, and on their amino acid compositions (Table I).

To our knowledge, the results presented herein represent the first report of the application of reversed-phase HPLC to separate different forms of apoferritin subunits in analytical or preparative amounts. The only other preparative means presented to date has been the use of electrochromatography by Otsuka and Listowsky[17] to separate limited quantities of "H" and "L" subunits. Certainly, reversed-phase HPLC represents an important tool for the study of ferritin structure and function. It offers the investigator the ability to separate completely milligram amounts of (at least) two different forms of ferritin subunits so that the actual chemical differences between the subunits species may then be elucidated.

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