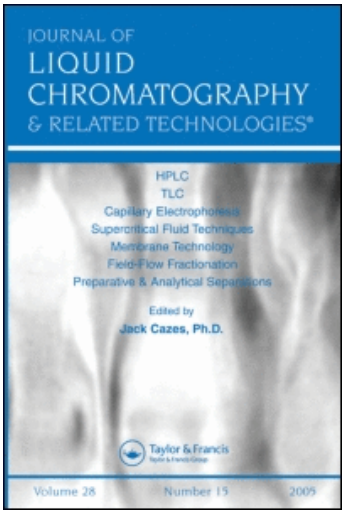

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Multiple Ferritin Subunit Types by Reversed-Phase HPLC

J. F. Collawn Jr.[a]; H. Donato Jr.[a]; E. Council[a]; W. W. Fish[a]
[a] Department of Biochemistry, Medical University of South Carolina, Charleston, South Carolina

To cite this Article Collawn Jr., J. F. , Donato Jr., H. , Council, E. and Fish, W. W.(1986) 'Separation of Multiple Ferritin Subunit Types by Reversed-Phase HPLC', Journal of Liquid Chromatography & Related Technologies, 9: 8, 1679 — 1688
To link to this Article: DOI: 10.1080/01483918608076711
URL: http://dx.doi.org/10.1080/01483918608076711

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(8), 1679-1688 (1986)

SEPARATION OF MULTIPLE FERRITIN SUBUNIT TYPES BY REVERSED-PHASE HPLC

J. F. Collawn, Jr., H. Donato, Jr.,
E. Council, and W. W. Fish
Department of Biochemistry
Medical University of South Carolina
Charleston, South Carolina 29425

ABSTRACT

Subjection of ferritin subunits to reversed-phase HPLC effected a separation of them into three chromatographic species. These three putative subunit species were found in varying proportions from porcine spleen, liver, and heart ferritins as well as human liver, spleen, and placental ferritins. Detailed chemical-physical characterization of each of the isolated species indicate them to be two H-type and one L-type ferritin subunits. Thus, reversed-phase HPLC presently stands as the only available means to separate preparatively the various subunit types of ferritin.

INTRODUCTION

The soluble iron storage protein, ferritin, is commonly found in both plants and animals (1). In animals, the richest sources of ferritin are spleen, liver, and bone marrow although most tissues contain ferritin. The structure of ferritin is rather unique in that it consists of a protein shell of twenty-four subunits arranged to form a hollow center which

 0148-3919/86/0908-1679$3.50/0

can contain up to 4,500 atoms of iron in the form of ferric oxide (1).

Selected denaturing polyacrylamide gel electrophoresis systems suggested that instead of being composed of identical subunits as originally believed, ferritin is composed of two subunit types (2). These two subunit types, designated H-type and L-type were observed to vary in relative amounts from tissue to tissue, with iron content, and with certain disease states (2). Because these two putative subunit types appeared to be so similar chemically and physically and because they could be separated only under selected electrophoretic conditions, the existence of two types of ferritin subunits was challenged for several years. In searching for a method capable of preparatively isolating these putative subunits in sufficient quantities for amino acid sequence determination, we employed reversed-phase HPLC and found it to provide resolution for analytical or preparative separations which far exceeds existing methodologies. This system, together with substantiation of its ability to separate different subunit species of ferritin are discussed in the following.

MATERIALS

Porcine ferritin was prepared from tissues obtained fresh at the abattoir. Purification procedures followed protocol described previously (3,4). Human liver and spleen tissues were obtained 12 hr. postmortem through the Department of Pathology, M.U.S.C. Ferritin isolation was initiated immediately. Human placentas were obtained at delivery and placed on ice; ferritin isolation was begun within two hours of obtaining the placentas. The purification procedures employed for ferritin isolation from human tissues have been described earlier (5).

A Synchropak RP-P column (C-18, 25 cm x 10 mm) was obtained from Synchrom, Inc. (Linden, IN). Acetonitrile

and water, both HPLC-grade, were obtained from J.T. Baker. Trifluoroacetic acid was obtained from Sigma.

METHODS

Ferritin subunits were prepared by performing simultaneous iron reduction and subunit dissociation via treatment of the ferritin with acetic acid-thioglycolic acid (4).

HPLC was performed on a Varian 5000 liquid chromatograph equipped with a variable wavelength detector. The solvent systems routinely employed were: solvent A, 0.1% trifluoroacetic acid in water and solvent B, 0.1% trifluoroacetic acid in acetonitrile. The following program was ultimately used for routine separation of the three subunit species: injection of the sample at 100% A, a linear gradient from 0% to 25% B in 1 min, a second linear gradient from 25% to 50% B over 44 min, and a final gradient from 50% to 80% over 5 min. The column flow rate was 3.0 ml/min.

All other methodology employed in this study is described in detail elsewhere (4,5,6).

RESULTS

Purified porcine spleen ferritin appeared homogeneous by disc gel electrophoresis of the native protein and sodium dodecyl sulfate polyacrylamide gel electrophoresis of the subunits (gels not shown). However, upon subjection of the porcine spleen ferritin subunits to reversed-phase HPLC, three chromatographic species were observed (Fig. 1A). Furthermore, each of the isolated chromatographic species rechromatographed as a single peak at the same binary solvent composition under which it originally eluted (Fig. 1B). Sedimentation velocity measurements were performed on each of the chromatographic species in solvent of the same composition as that at which the chromatographic species eluted during reversed-phase HPLC. At a protein concentration of 1 mg/ml, the sedimentation coefficients of the three species

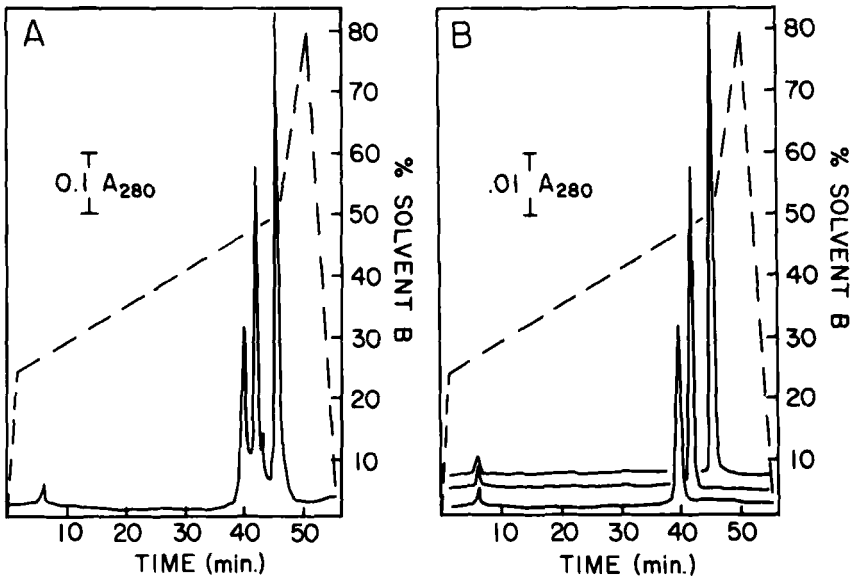

FIGURE 1. The separation of porcine spleen ferritin subunits by reversed-phase HPLC. Experimental details are provided in the text. A. Porcine spleen ferritin subunits. B. Re-chromatography of each chromatographic species from (A).

were the same ($s_{20,w}$ = 1.0 ± 0.1 S). This virtually rules out the possibility that under the solvent conditions utilized for the gradient elution during HPLC, one (or more) of the three chromatographic species might represent an aggregated form of the other species. The measured sedimentation coefficient is consistent with a partially unfolded (or in some way extended) form of monomeric subunit of molecular weight ∿20,000.

We have also found that the use of 2-propanol instead of acetonitrile, together with minor modifications in the HPLC elution program, also effects separation of the same three chromatographic species. Thus, the three species do not appear to be an artifact of the organic solvent employed in the binary system.

As a second means to examine the authenticity of the three chromatographic species of ferritin subunits, we tested for a tissue-specific change in the ratio of the three putative subunit types. This approach was used by others who employed polyacrylamide gel electrophoresis under denaturing conditions to monitor for differences (2). We applied this approach to porcine ferritins which were isolated from the spleen, liver, and heart of a single animal. Again, three chromatographic species were observed when subunits prepared from each of the tissue ferritins were subjected to reversed-phase HPLC (4). The distribution of protein among the three chromatographic fractions changed, in turn, with the tissue source of the ferritin (Table 1). It can be seen that HPLC species 1 and 2 predominated in heart while species 3 was greatest in spleen. Within experimental error, it also appears that the proportion of species 1 relative to species 2 remained constant regardless of the percent of the total ferritin subunit population constituted by those two subunit species (Table 1).

Because most of the experimental observations regarding porcine ferritin were limited to those from our laboratory, tissue ferritins from a more widely studied source, human, were examined in a manner similar to that described above. This provided a broader base of experimental results with which to compare our analyses by reversed-phase HPLC. Human spleen and liver ferritins were purified from the tisssues of a single individual (5) while human placental ferritin was purified from a collection of full term placentas. Subunits prepared from the ferritin isolated from each of these tissues were subjected to reversed-phase HPLC. As was observed for porcine ferritin, three chromatographic species were observed. Additionally, the individual chromatographic species were present in different ratios in the three tissues (Table 1).

TABLE 1

Variation In Ferritin Subunit Types From Human and Porcine Tissues

Tissue Source	% Peak 1	% Peak 2	% Peak 3	$\frac{\% \text{ Peak 1} \times 100}{\% \text{ Peak 1} + \% \text{ Peak 2}}$
Porcine spleen	22	28	50	44
Porcine liver	19	42	39	31
Porcine heart	34	61	5	36
Human spleen	9	16	75	36
Human liver	12	20	68	38
Human placenta	23	33	44	41

In an attempt to determine if the three chromatographic species which were observed for ferritin subunits were indeed each chemically unique, we examined a number of physical and chemical properties of each. Table 2 summarizes the results for porcine spleen ferritin subunits (6). As can be seen, all three putative subunit species are the same molecular weight and possess little or no covalently attached carbohydrate. Although complicated by the fact that ferritin subunits tend to aggregate in all isoelectric focusing systems tried to date (7), the focusing pattern of species 1 and 2 were quite similar and were distinct from that of species 3 (6). Comparison of the amino acid compositions of the three subunit chromatographic species (6) indicated no statistically significant compositional differences between species 1 and 2. However, several distinct compositional differences were noted when these two species were compared to subunit 3. These amino acid compositional similarities and differences are also reflected in the calculated HPLC-retention constants (8) of the three species in that

TABLE 2

A Chemical and Physical Comparison of Porcine Ferritin Subunit Species Separated by Reversed-Phase HPLC

Chemical/Physical Property	Magnitude of the Property		
	Species 1	Species 2	Species 3
Molecular Weight	19,800	19,800	19,600
Covalently-Attached Carbohydrate	<0.3%	<0.3%	<0.3%
pI	5.1-5.9	5.1-5.9	5.7-6.2
Reversed-Phase HPLC Retention Constant Predicted from Amino Acid Composition (8)	84.2	85.9	90.4

the empirically estimated constants are consistent with the sequence of elution from the reversed-phase column (Table 2; cf Fig. 1). In an effort to obtain unequivocal evidence for the chemical individuality of each of the three ferritin subunit chromatographic species, we have initiated the determination of the amino acid sequence of each of the chromatographic species obtained from porcine spleen ferritin. Comparison of the preliminary amino acid sequence information obtained for corresponding regions of each of the three chromatographic species clearly indicated differences between the amino acid sequence of species 3 and those of species 1 and 2. No sequence differences between species 1 and 2 have yet been observed; however, a very high degree of homology between these two protein species is predicted by their similar behavior during reversed-phase HPLC.

DISCUSSION

For over a decade, a debate ensued over whether the iron storage protein, ferritin, was composed of more than one type of subunit. The multiple forms, if they existed, were of the same size, possessed very similar charge properties, and possessed quite similar amino acid compositions. Thus, it had not been possible to separate these putative subunit types by the more conventional biochemical means of protein purification. The major impact of the work presented herein is the demonstration that indeed more than one type of ferritin subunit composes functional ferritin and that reversed-phase HPLC is the only method to date which can separate these subunits in preparative quantities. This is most clearly illustrated by the data for porcine ferritin. Reversed-phase HPLC separates three populations of ferritin subunits from material which gives only one band with sodium dodecyl sulfate polyacrylamide gel electrophoresis, the analytical method which first gave rise to the "isosubunit" model for ferritin (9). Additionally, our results for human ferritins support the results for porcine ferritin and also amplify observations by other methodologies (2).

The recent isolation of cDNA clones for two different human ferritin chains further substantiate the existence of multiple ferritin subunit types (10,11,12,13). Furthermore, genomic analyses of these cDNAs suggest that ferritin subunits are encoded by two multi-gene families (12,13). The physiological significance of these multi-gene families is presently not understood. However, with the ability to separate the products of expression by these genes through the use of reversed-phase HPLC, we now stand ready to elucidate the physiological and structural bases for the unique functional and metabolic characteristics of each of the ferritin subunit types.

ACKNOWLEDGEMENTS

This research was supported in part by USPHS grant HL-19491 to W.W.F. and in part by funds from the M.U.S.C. Summer Undergraduate Research Faculty Program to H.D., Jr. This research is also part of a dissertation to be submitted by James F. Collawn, Jr. to the Graduate College of the Medical University of South Carolina in partial fulfillment of the requirements for the Ph.D. degree.

REFERENCES

1. Aisen, P. and Listowsky, I., Iron Transport and Storage Proteins, Ann. Rev. Biochem., 49, 357, 1980.

2. Drysdale, J.W., Adelman, T.G., Arosio, P., Casareale, D., Fitzpatrick, P., Hazard, J.T. and Yokota, M., Human Isoferritins in Normal and Disease States, Semin. Hematol., 14, 71, 1977.

3. May, M.E. and Fish, W.W., The Isolation and Properties of Porcine Ferritin and Apoferritin, Arch. Biochem. Biophys., 182, 396, 1977.

4. Collawn, J.F. Jr. and Fish, W.W., The Distribution of Ferritin Subunit Types in Porcine Tissues, Comp. Biochem. Physiol., 78B, 653, 1984.

5. Collawn, J.F., Jr., Donato, H. Jr., Upshur, J.K. and Fish, W.W., A Comparison by HPLC of Ferritin Subunit Types in Human Tissues, Comp. Biochem. Physiol., 81B, 901, 1985.

6. Collawn, J.F. Jr., Lau, P.Y., Morgan, S.L., Fox, A. and Fish, W.W., A Chemical and Physical Comparison of Ferritin Subunit Species Fractionated by High-Performance Liquid Chromatography, Arch. Biochem. Biophys., 233, 260, 1984.

7. Watanabe, N. and Drysdale, J., Studies on Heterogeneity in Ferritin Subunits, Biochim. Biophys. Acta, 743, 98, 1983.

8. Sasagawa, T., Okuyama, T. and Teller, D.C., Prediction of Peptide Retention Times in Reversed-Phase High-Performance Liquid Chromatography During Linear Gradient Elution, J. Chromatogr., 240, 329, 1982.

9. Adelman, T.G., Arosio, P. and Drysdale, J.W., Multiple Subunits in Human Ferritins: Evidence for Hybrid Molecules, Biochem. Biophys. Res. Commun., 63, 1056, 1975.

10. Costanzo, F., Santoro, C., Colantuoni, V., Bensi, G., Raugei, G., Romano, V. and Cortese, R., Cloning and Sequencing of a Full Length cDNA coding for a Human Apoferritin H Chain: Evidence for a Multigene Family, EMBO J., 3, 23, 1984.

11. Boyd, D., Jain, S.K., Crampton, J., Barrett, K.J. and Drysdale, J., Isolation and Characterization of a cDNA Clone for Human Ferritin Heavy Chain, Proc. Natl. Acad. Sci. USA, 81, 4751, 1984.

12. Dorner, M.H., Salfeld, J., Will, H., Leibold, E.A., Vass, J.K. and Munro, H.N., Structure of Human Ferritin Light Subunit Messenger RNA: Comparison with Heavy Subunit Message and Functional Implications, Proc. Natl. Acad. Sci. USA, 82, 3139, 1985.

13. Boyd, D., Vecoli, C., Belcher, D.M., Jain, S.K. and Drysdale, J.W., Structural and Functional Relationships of Human Ferritin H and L Chains Deduced from cDNA Clones, J. Biol. Chem., 260, 11755, 1985.