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Ox Spleen Ferritin: an Isoelectrofocusing and Crossed Immunoelectrofocusing Study

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Ox spleen ferritin was purified and its purity checked by two-dimensional immunoelectrophoresis and polyacrylamide plate electrophoresis. Microheterogeneity was shown with a preparation of purified ferritin by isoelectric focusing. The protein was separated into at least 6 fractions; two large fractions in the 4.50-4.55 pH range and another 4 in the 4.65-4.80 interval. Microheterogeneity was confirmed in purified preparations by crossed immunoelectrofocusing. Seven fractions were observed, the most acid ones (4.50-4.55) also being the most abundant. In the crossed IEF procedure, exactness in the isoelectrophoretic separation time is important in that excessive time may impair the resolution potential.

Key words: Ferritin, Isoelectrofocusing, Ferritin heterogeneity.

Ferritin, an iron protein, is widely distributed throughout the animal, plant and bacterial kingdoms. In mammals, the ferritins of different organs have been characterized —heart, liver, spleen, kidney— in species such as rat (4), pig (8), sheep (10), horse (13) and man (14).

The ferritin molecule comprise a protein structure of 480 Kd made up of 24 subunits with an approximate molecular weight of 20 Kd and nucleus of varying size (13). Ferritins of different organs exhibit differing electrophoretic behaviour, as has been shown in isoelectrofocusing experiments (2). The origin of heterogeneity of ferritin lies in the existence of two kinds of subunits; H and L (21 Kd and 19 Kd, respectively) (1), which are joined in different combinations to constitute different isoferritins (1, 13). These subunits are the products of different genes (13).

By the use of isoelectrofocusing techniques it is possible to characterize the isoferritins of diverse animal species; in the present work ox spleen ferritins are characterized by isoelectrofocusing and crossed immunoelectrofocusing. The latter technique was employed because it provides better resolution.

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Materials and Methods

Isolation of ox-spleen ferritin. — This was obtained from spleen tissue according to the procedure described by MAY and FISH (8) for pig spleen ferritin.

Electrophoretic characterization of the ferritin. — The ferritin from ox-spleen was characterized by two electrophoretic techniques: *a*) Polyacrylamide plate electrophoresis, based on the technique of ORSTEIN (11). The sample, 10 µg of ferritin, was applied to the gel electrophoresis apparatus; the plates were stained with Comassie blue R-250, specific to proteins, and potassium ferrocyanide at 0.2 % in 3 % trichloroacetic acid, specific to iron. *b*) Two- dimensional immunoelectrophoresis; this was performed according to the technique of LAURELL (6). For the first electrophoretic separation, 10 µg of purified ferritin were used which in the second separation were run against specific antiserum obtained from rabbits at 2.3 %. Sigma type I agarose gel was employed.

Polyacrylamide gel isoelectrofocusing (PAGIF). — Isoelectrofocusing was carried out on thin layer polyacrylamide gels (PAGIF). Gels (80 × 80 × 1 mm) were prepared from 6 ml acrylamide, bis acrylamide solution (9.70 and 0.30 %, respectively) 0.37 ml ampholyte, pH 2.5-5.0; 0.37 ml ampholyte, pH 5-8 (Pharmacia); 1.2 g sucrose and 3.64 ml of distilled water. Air bubbles were removed from the mixture by applying vacuum for 20 min, 180 µl ammonium persulphate (1 %) was added. The mixture was loaded into a prepared gel mold with a syringe, and the fels polymerized within 30 min; 10 µl ferritin (12 µg) were applied to a filter paper and placed on the gel at 2 cm from the cathodic edge of the plate. A Pharmacia FBE 3000 multiphor chamber was used. Isoelectrofocusing was performed for 60 min at 16 W up to maximum of 2,000 V; 0.1 M L-histidine and 0.03 M glutamic acid were employed at the cathode and anode, respectively. Protein fixing was accomplished by precipitation with sulphosalicylic acid and trichloroacetic acid, 30 min. Gels were stained with briant Comassie blue R-250 and the ferrocyanide method.

Determination of isoelectric points. — The isoelectric points (IP's) of the ferritins separated by isoelectrofocusing were determined by comparison with markers of known IP: methyl red - 3.75; glucose oxidase - 4.5; soybean trypsin inhibitor - 4.55; B-lactoglobulin - 5.20 and anhydrase B - 5.85 (Pharmacia).

Crossed immunoelectrofocusing (IEF). Isoelectrofocusing was carried out in agarose IEF gel (Pharmacia). Gels (90 × 110 × 1 mm) were prepared from 0.17 g IEF agarose, 2.04 g D-sorbitol and 15.30 ml distilled water. The mixture was heated until it dissolved. Following this it was allowed to cool to about 75°C and 0.5 ml of ampholyte 2.5-5.0 and 0.5 ml of ampholyte 5.0-8.0 (Pharmacia) were added, 10 µl ferritin (12 µg) were applied to a filter paper and placed on the gel at 2 cm from the cathodic edge of the plate. IEF was performed several times at 7 W up to maximum of 1.500 V; times ranged between 1 h 26 min and 2 h 50 min.

Once the first dimension had been performed, the ferritins were run against the specific anti-rabbit antiserum at 3.3 % in immunoelectrophoresis. Finally the plates were stained with Comassie blue R-250.

Results

The polyacrylamide gel electrophoresis of ox spleen ferritin (fig. 1) exhibits one monomer and one dimer. Two-dimensional immunoelectrophoresis of the protein isolated revealed only one precipitin arc (fig. 2).

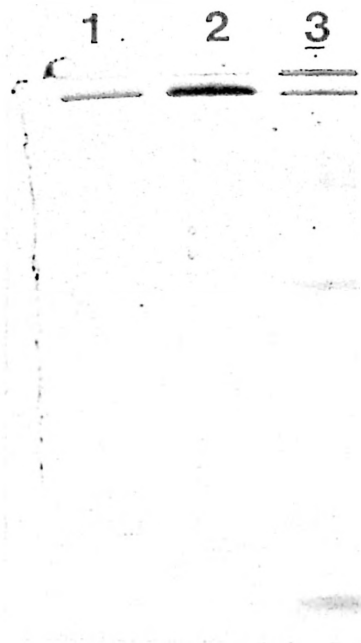

Fig. 1. *Polyacrylamide plate electrophoresis of extract of purified ox-spleen ferritin.*
Lane 1: 10 μg; Lane 2: 20 μg; Lane 3: markers of molecular weight.

When ox spleen ferritin was subjected to PAGIF (fig. 3) several proteins could be observed located at a pH range of 4.5-4.8; in particular it was possible to observe in the photography 8 bands grouped

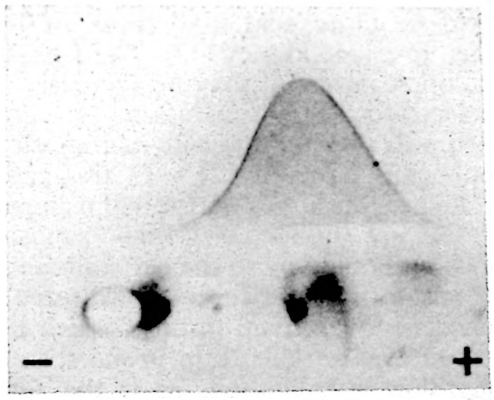

Fig. 2. *Two-dimensional immunoelectrophoresis of ox-spleen ferritin.*

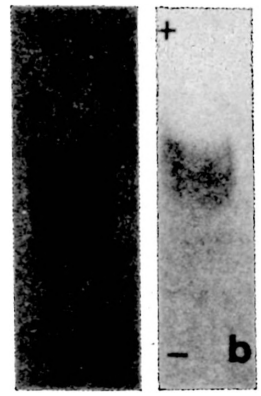

Fig. 3. *Polyacrylamide plate isoelectrofocusing of ox-spleen ferritin.*
a) Coomassie blue staining, *b*) specific staining for iron.

into 4 fractions. Their IP's were determined by comparison with standards of known IP's. The more acid isoferritins appear as thicker bands compared with others. Iron specific staining gave identical results.

The crossed IEF study of the purified fraction (fig. 4) carried out under the same isoelectrophoretic conditions shows the best resolution, according to the different times employed, after 1 h 54 min. With this technique it is possible to distinguish 7 immunoprecipitations corresponding to the same number of fraction.

Discussion

Until now, isoelectrofocusing has often been used to correlate the isoelectric point of the different isoferritins to their iron content (1-4, 15, 16), their subunit composition (1-4, 15) and even to the incorporation kinetics of iron within the protein cavity (12, 14, 15). In this sense, it may be considered as a particularly valuable technique for the characterization and differentiation of the ferritins from different organs of a particular species. The good resolution obtained with isoelectrofocusing in polyacrylamide gels as

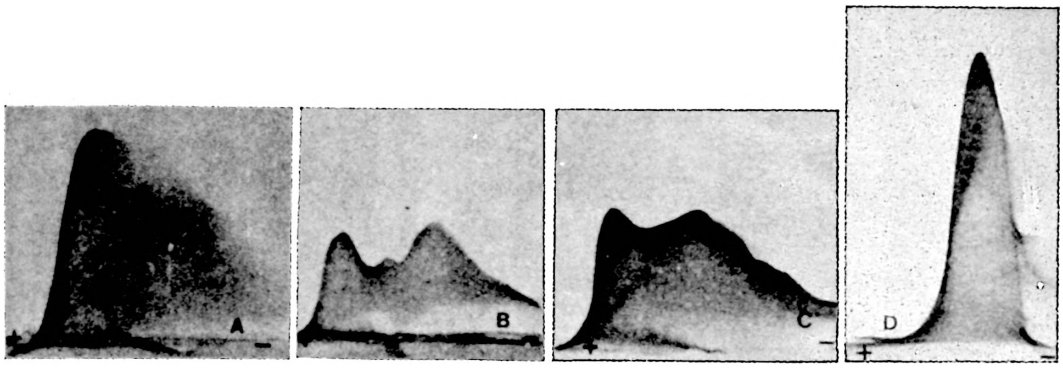

Fig. 4. *Crossed immunoelectrofocusing of purified ox-spleen ferritin after different isoelectrofocusing separation times.*
A: 1 h 20 min; B: 1 h 35 min; C: 1 h 54 min; D: 2 h 45 min.

compared with other analytical techniques (electrophoresis) permits the separation of proteins into molecular species of different IP's: it is furthermore possible to detect differences as small as 0.01 U in the IP's. Accordingly, even though two protein molecules may have the same M.W. and may display the same electrophoretic migration patterns it is unlikely that their charges will be identical and it is therefore possible to differentiate between them with this technique. Having ox-spleen ferritin, the purity of preparation was checked by electrophoresis on polyacrylamide gel plates; the typical electrophoretic pattern of purified ferritin could be observed. Furthermore, a second criterion for purity (two-dimensional electrophoresis) gave only a single precipitin arc, clearly evidencing the purity of the preparation. The molecular species of ox-spleen ferritin analyzed by PAGIF are acid; the IP's range from 4.40 to 4.80. Six fractions may be distinguished, comprising 12 bands. These results coincide with those of WAGSTAFF *et al.* (15) in which for human hepatic ferritin 6 fractions are detected, each containing more than one band. The application of PAGIF to the detection of isoferritins has been employed since the middle of the sixties; however, the technique has been gradually modified in order to increase the power of resolution, mainly by increasing the wattage and decreasing the time of electrofocusing, which has changes from 3 W and 18 h to 10 W and 1 h for a single plate, in our study. However, it has not been possible to achieve sufficient resolution to permit the exact visualization of a number of bands; in rat heart ferritin and liver ferritin 6-7 bands are detected (4); in horse heart ferritin, 15-18 bands (12); in horse spleen ferritin, 11-15 bands (12). In the present work 12 bands in 6 fractions were visualized which after photography are difficult to observe totally. These results are in agreement with others in which spleen ferritins are reported to be more acid than those of the heart, liver and kidney (12). The PAGIF resolution potential may be increased if isoelectrofocusing separation is combined with a second run with immunoelectrophoresis (crossed IEF). In this case, the first separation is performed using an agarose gel (of low electroendosmosis) which upon binding to the gel containing the anti-ferritin antiserum constitutes a homogeneous medium through which the passage of electric current in the second dimension is facilitated, as is the final separation. Once the isoelectrofocusing conditions have been determined —pH

range, temperature, wattage, etc.—(common to all separation by IEF), the resolution potential by crossed IEF is time-conditioned. As may be seen in figure 4, 1 h 54 min is needed to achieve the best resolution. Seven precipitin arcs may be visualized, corresponding to the same number of ferritin fractions. The semi-quantitative nature of the technique permits the identification of those ferritins which appear at the highest concentrations and their relationship with the IP; it may be seen that the two most acid fractions (IP's 4.40 and 4.55) are also the most abundant. This has already been observed with PAGIF. Moreover, with this technique it is easy to detect on a single plate the immunorreactive characteristics of different types of ferritin against different specific antibodies, making it possible to characterize the immunological properties of each isoferritin without the need to separate them by preparative electrofocusing, as has been the case to the present (15), and then applying RIA techniques with different specific antibodies. Furthermore, it is not necessary to use purified and radioactively labelled antibodies to immunologically characterize the different isoferritins, as has been described in other works (5, 9). Finally, the application of crossed IEF points clearly to the heterogeneity of ox-spleen ferritin as compared with that of other species and may permit the establishment of a hypothesis concerning the genetic control of other proteins in which it has been possible to demonstrate the existence of pathological alleles (7). In conclusion, the application of these techniques could contribute to a better knowledge concerning the structure and physiology of this important protein.

Resumen

Se aísla ferritina de bazo de toro comprobándose su pureza por electroforesis en placa de poliacrilamida y por inmunoelectroforesis bidimensional; su microheterogeneidad se ha puesto de manifiesto por isoelectroenfoque: la proteína se ha separado en seis fracciones: dos grandes fracciones de pH entre 4,50-4,55 y otras cuatro en el intervalo 4,65-4,80. La microheterogeneidad fue confirmada por inmunoelectroenfoque cruzado, observando siete fracciones, siendo las más ácidas las más abundantes (pH 4,50-4,55). En el procedimiento de inmunoelectroenfoque cruzado es importante la exactitud en el tiempo de la separación, ya que un tiempo excesivo puede disminuir la capacidad resolutiva de esta técnica.

References

1. Arosio, P., Adelman, T. G. and Drysdale, J. W.: *J. Biol. Chem.*, 253, 4451-4458, 1978.
2. Bomford, A., Lis, Y., Macfarlane, I. G. and Williams, R.: *Biochem. J.*, 167, 309-312, 1977.
3. Bomford, A., Berger, M., Lis, Y. and Williams, R.: *Biochem. Biophys. Res. Commun.*, 83, 334-341, 1978.
4. Bomford, A., Conlon-Hollingshead, Ch. and Munro, H. N.: *J. Biol. Chem.*, 256, 948-955, 1981.
5. Halliday, J. W., Mack, U. and Powell, L. W.: *Arch. Intern. Med.*, 138, 1109-1113, 1978.
6. Laurell, C. B.: *Anal. Biochem.*, 10, 358-363, 1965.
7. Leone, G., Valori, V. M., Contumaccio, R. and Massi, G.: *Haemostasis*, 13, 369-373, 1983.
8. May, M. E. and Fish, W. W.: *Archs. Biochem. Biophys.*, 182, 396-403, 1977.
9. McKeering, L. W., Halliday, J. W. and Caffin, J. A.: *Clin. Chim. Acta*, 67, 189-197, 1976.
10. Mertz, J. R. and Theil, E. C.: *J. Biol. Chem.*, 258, 11719-11726, 1983.
11. Ornstein, L.: *Ann. NY. Acad. Sci.*, 121, 321-349, 1964.
12. Russell, S. M. and Harrison, P. M.: *Biochem. J.*, 175, 91-104, 1978.
13. Stefanini, S., Chiancone, E., Arosio, P., Finazzi-Agro, A. and Antonini, E.: *Biochemistry*, 21, 2293-2299, 1982.
14. Wagstaff, M., Worwood, M. and Jacobs, A.: *Biochem. J.*, 173, 969-977, 1978.
15. Wagstaff, M., Worwood, M. and Jacobs, A.: *Clin. Sci.*, 62, 529-540, 1982.
16. Worwood, M., Dawkins, S., Wagstaff, M. and Jacobs, A.: *Biochem. J.* 157, 97-103, 1976.