PREPARATIVE ELECTROPHORETIC DESORPTION IN THE PURIFICATION OF HUMAN SERUM FERRITIN BY IMMUNO-ADSORPTION

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1. Introduction

Since the original purification of ferritin by Laufberger [1] using cadmium salt precipitation a number of purification techniques have been described using various combinations of heat denaturation and differential precipitation with ammonium sulphate together with ultra-centrifugation and various forms of column chromatography [2,3]. The disadvantages of these methods are two-fold. Firstly, they usually involve several stages during which protein denaturation may occur and secondly, the yield is often very low, the latter being a major problem when working with a source of ferritin of low concentration, e.g., normal serum. Furthermore, ultra-centrifugation tends to select only those ferritin molecules with a relatively high iron content.

This paper describes a method of ferritin purification which can be applied to both tissue and serum ferritin and combines the advantages of a one-step purification with high yield of recovered ferritin. The method involves an adsorption phase using a specific immuno-adsorbent combined with a previously described preparative application of electrophoretic desorption [4]. This desorption method was compared with more conventional methods.

2. Methods and materials

Anti-human ferritin antibodies were raised in New Zealand white rabbits against human spleen ferritin which had been purified and assessed by established methods [5]. A horse ferritin cellulose based immuno-adsorbent [6] was originally used to purify ferritin antibodies from whole rabbit serum. At a later stage rabbit and human ferritin anti-sera were prepared which did not react with the immuno-adsorbent. The latter antisera were Rivanol-treated before use to remove protein other than γ-globulins [7]. Ferritin concentrations were determined by an immunoradiometric assay [5]. Polyacrylamide gel electrophoresis was performed on 7% polyacrylamide disc gels [8]. Electrophoresis was carried out at room temperature with a constant current density of 5 mA/gel for 1–1.5 h.

2.1. *Preparation of affinity matrix*

Sepharose 4B (Pharmacia, Croydon, England) was activated with cyanogen bromide as described for Ultrogel® by Doley et al. [9]. A solution of anti-ferritin antibody (1 ml) containing 337 μg protein was incubated with the cyanogen bromide-activated agarose (2 g) at 4°C for 24 h. The antibody matrix complex was washed with 0.01 M sodium phosphate buffered saline (PBS) pH 7.4 until no protein could be detected in the supernatant. The coupling efficiency was 95%. The antibody–matrix complex was then washed with a large volume of 6 M guanidine hydrochloride (pH 3.1) and subsequently with excess of PBS buffer before use.

2.3. *Adsorption and desorption*

Serum (1 L), normal or from a patient with haemochromatosis undergoing therapeutic venesection was incubated with the immunoadsorbent for 2 h at 4°C with continuous mixing. A solution of human spleen ferritin of known concentration was also

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adsorbed as a control. The charged immunoadsorbent was filtered off, packed into a small chromatography column (1 × 20 cm) and washed with PBS until the optical density was zero.

2.4. *Method A*

Ferritin adsorbed to the affinity matrix was then eluted with 6 M guanidine HCl (pH 3.1). Fractions (0.5 ml) containing protein were collected and dialysed against 0.1 M sodium acetate at 4°C for 24 h and then exhaustively against 0.01 M PBS. The protein containing solution was assayed for ferritin content [5].

2.5. *Method B*

The charged immunoadsorbent was prepared as above and washed with 0.3 M borate buffer, pH 8.6. The lower end of the column was closed with a dialysis membrane and placed in a buffer reservoir. The upper end of the column was fitted with a second buffer reservoir. A voltage was then applied to the column to maintain a constant current density of 15 mA using platinum electrodes placed in the buffer reservoirs with the anode in the lower chamber. After 3 h at room temperature ferritin was recovered from the chamber (500 μl) above the dialysis membrane. This solution was assayed for ferritin content as above.

2.6. *Method C*

I A glass tube was prepared as for polyacrylamide gel electrophoresis. 40% sucrose solution (100 μl) was added, on top of which a polyacrylamide gel (5 × 20 mm) was formed. After polymerisation the sucrose solution was removed, replaced with 50 mM Tris–glycine buffer, pH 8.3, and the end sealed with a dialysis membrane. The tube was fitted to a polyacrylamide disc-gel electrophoresis tank (Shandon) and the buffer reservoir filled with the Tris–glycine buffer. The charged matrix was pipetted onto the top of the polyacrylamide gel and a current density of 5 mA maintained for 1 h. The protein containing solution was recovered from the chamber above the dialysis membrane and analysed by polyacrylamide gel electrophoresis for protein and iron.

II At this point a second adsorption was performed. The desorbed protein was readsorbed by recycling through a column of the same immunoadsorbent (5 × 20 mm) at room temperature for 2 h. The recharged immunoadsorbent was then electrophoretically desorbed as above (Method C (I)). The recovered protein was analysed by polyacrylamide gel electrophoresis.

Table 1
The recovery of ferritin by different elution procedures

	Pre-adsorption	Post-adsorption	Eluate	% Expected Yield
Guanidine elution (Method A)				
Human Spleen Ferritin	1040	787	252.5	100
Norman Human Serum	43.4	0.5	42.9	99
Haemochromatotic Serum	820	720	100	40
Electrophoretic desorption (Method B)				
Human Spleen Ferritin	1040	792	248	100
Normal Human Serum	35.6	0.5	34.5	97
Haemochromatotic Serum	380	145	235	95

% Expected yield for human spleen ferritin refers to the maximum amount of ferritin adsorbed from excess ferritin by the immunoadsorbent (2 g)

The capacity of the immunoadsorbent, 125 μg ferritin/g immunoadsorbent

The % expected yield for the serum extracts refers to the total amount of ferritin available or the maximum immunoadsorbent capacity if the total ferritin exceeds this

3. Results

3.1. *Recovery of ferritin*

Table 1 shows the ferritin content of samples both pre- and post adsorption together with the ferritin content of the eluate determined by immunoradiometric assay. Greater than 95% of both normal and haemochromatotic serum ferritin was adsorbed and successfully eluted by both methods (except in the guanidine elution of haemochromatotic serum ferritin where the immunosorbent had been used in excess of five times and had become less active). The ferritin determinations were made soon after elution, nevertheless guanidine-eluted protein was shown to be unstable and precipitated on standing, therefore this method was discontinued.

3.2. *Assessment of purity*

Figure 1a shows the polyacrylamide gel electrophoretic pattern obtained from the electrophoretically desorbed (Method C (I)) human spleen ferritin stained for iron with potassium ferocyanide and for protein with Coomassie blue. Both the monomeric and dimeric forms of ferritin were present and furthermore the protein bands stained for iron.

Figure 1b shows the gel electrophoretic pattern of the normal human serum extract prepared by method C (I). This is compared to normal human serum. A ferritin protein ratio of about 1:40 was found.

Figure 1c shows the gel electrophoretic pattern of the serum extract after the second adsorption and subsequent electrophoretic desorption (Method C (II)). A single band of monomeric ferritin was present which did not stain for iron.

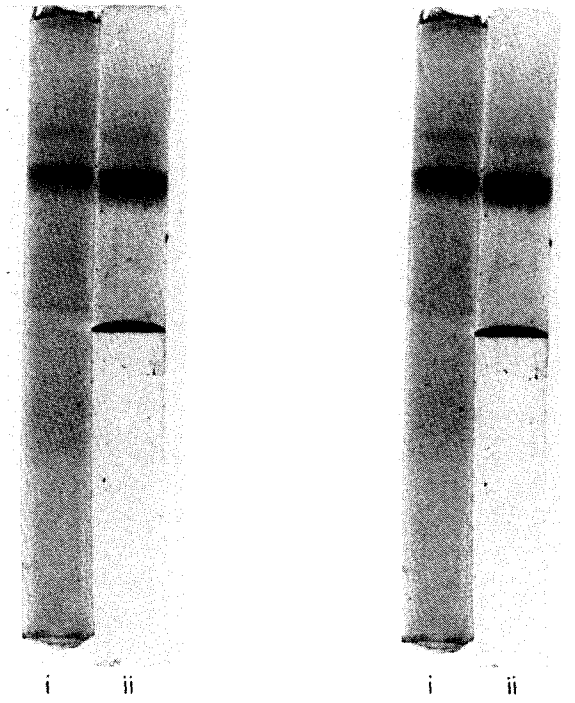

Fig.1a

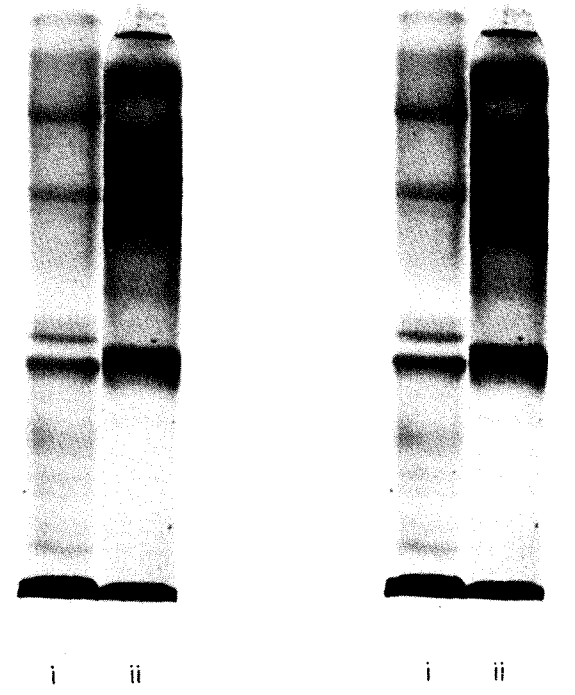

Fig.1b

4. Discussion

Previous attempts at the purifications of serum ferritin have relied on conventional techniques usually employed in the extraction of tissue ferritins where the ferritin concentration is high and the losses during purification are of little concern [10]. We have shown that immunoadsorption is a rapid and sensitive technique capable of extracting small quantities of ferritin from solutions of low ferritin concentration and high non-ferritin protein content with little or no losses and was particularly suitable in the purification of serum ferritin. The technique of electrophoretic

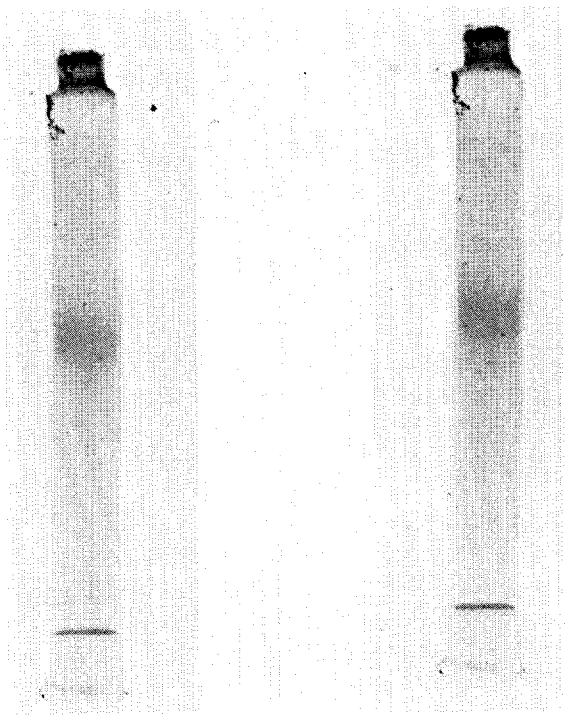

Fig.1c

Fig.1a. Polyacrylamide gel electrophoresis of purified human spleen ferritin following electrophoretic desorption (Method C (I)) and stained for iron (Gel i) and protein (Gel ii).

Fig.1b. Polyacrylamide gel electrophoresis of human serum ferritin (purified by Method C (I)), (Gel ii) compared with whole serum (Gel i) and stained for protein.

Fig.1c. Polyacrylamide gel electrophoresis (stained for protein) of purified human serum ferritin using Methods C (I) and C (II).

desorption allows the ferritin to be recovered in small volumes with no evidence of desorption damage, the final extract having a high degree of purity.

The application of affinity chromatography with electrophoretic desorption to the problem of isolating tumor specific isoferritins from the serum of patients with neoplastic disease needs to be assessed. The iron storage protein ferritin is usually present in serum at levels which reflect body iron stores [11]. In malignant disease the level is often raised [12–15] and it seems likely that at least some of the raised serum ferritin is derived from tumour cells [16]. Isoelectric focusing has demonstrated acidic isoferritins which may be tumour specific [17] and affinity chromatography with electrophoretic desorption may prove to be a useful method of selectively isolating these isoferritins in concentration sufficient for further analysis.

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References

[1] Laufberger, M. V. (1937) Bull. Soc. Chim. Biol. 19, 1575–1582.
[2] Linder, M. C. and Munro, H. N. (1972) Anal. Biochem. 48, 266–278.
[3] Penders, T. J., De Rooji-Dijk, H. H. and Leijnse, B. (1968) Biochim. Biophys. Acta 168, 588–590.
[4] Dean, P. D. G., Brown, P. J., Leyland, M. J., Watson, D., Angal, A. and Harvey, M. J. (1977) Biochem. Soc. Trans. 5, 1111–1113.
[5] Addison, G. M., Beamish, M. R., Hales, C. N., Hodskins, M., Jacobs, A. and Llewellin, P. (1972) J. Clin. Path. 25, 326–329.
[6] Miles, L. E. M. and Hales, C. N. (1968) Biochem. J. 108, 611–618.
[7] Exley, D., Johnson, M. N. and Dean, P. D. G. (1971) Steroids, 18, 605–620.
[8] Davies, B. J. and Ornstein, L. (1968) in: Methods in Immunology and Immunochemistry II (Williams, C. A. and Chase, M. V. eds) pp. 38–47.
[9] Doley, S. G., Harvey, M. J. and Dean, P. D. G. (1976) FEBS Lett. 65, 87–91.
[10] Worwood, M., Dawkins, S., Wagstaf, M. and Jacobs, A. (1976) Biochem. J. 157, 97–103.
[11] Jacobs, A., Millar, F., Worwood, M., Beamish, M. R. and Wardrop, C. A. (1972) Brit. Med. J. 4, 206–208.
[12] Jones, P. A. E., Millar, F. M. and Jacobs, A. (1973) Br. J. Cancer 27, 212–217.
[13] Marcus, D. M. and Zinberg, N. (1975) J. Nat. Cancer Inst. 55, 791–795.
[14] Niitsu, Y., Kongo, Y., Yokota, M. and Urushizaki, I. (1975) Ann. NY Acad. Sci. 259, 450–452.
[15] Mori, W., Asakawa, H. and Tazuchi, T. (1975) J. Nat. Cancer Inst. 55, 513–518.
[16] White, G. P., Worwood, M., Parry, D. and Jacobs, A. (1974) Nature 250, 584–586.
[17] Powell, L. W., Halliday, J. W. and McKeering, L. V. (1975) in: Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crichton, R. R. ed) pp. 215–221, North-Holland, Amsterdam.