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Note

The preparative separation of horse and human ferritins by chromatofocusing

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Chromatofocusing is a new convenient preparative chromatographic technique for separating proteins according to their isoelectric points. This method requires a simple chromatographic apparatus compared with the rather specialised equipment for preparative isoelectric focusing which has been used for several years to separate proteins with respect to their p*I* values.

Polybuffer (PB 74) and Polybuffer exchanger (PBE 94) have been designed by Pharmacia (Uppsala, Sweden) for chromatofocusing[1]. We investigated the application of this technique to the purification of ferritin, an iron storage protein, from horse and human spleen and liver preparations. We also compared it with gel filtration on Sepharose 6B, often used in ferritin purification[2].

EXPERIMENTAL

Polybuffer (PB 74), Polybuffer exchanger (PBE 94) and Sepharose 6B were purchased from Pharmacia (Hounslow, U.K.). Histidine and dithiothreitol were obtained from Sigma (Poole, U.K.) and ampholytes from LKB Instruments (South Croydon, U.K.). All other chemicals were AR grade or the highest grade available from BDH Chemicals (Poole, U.K.) or Fisons (Loughborough, U.K.) Ferritin was prepared from horse and human tissues by a procedure involving heat treatment at 70–75°C for 5 min and two precipitation steps with ammonium sulphate to 50% saturation (310 g/l). Samples were then dialysed against start buffer (0.025 *M* histidine–HCl pH 6.2 containing 1 m*M* dithiothreitol) before application to the PBE 94 column (25 × 0.9 cm I.D.), previously equilibrated with start buffer. The column was washed with this buffer until the absorbance of the eluting fractions, measured at 280 nm, approached zero. The pH gradient (pH 6.2 to 4.0) was formed by the application of 200 ml of PB 74 (diluted 1:8 with distilled water) adjusted to pH 4.0 with hydrochloric acid. A flow-rate of 25 ml/h was maintained by an LKB Varioperpex II peristaltic pump. Fractions (2 ml) were monitored by measuring the absorbance at 280 and 420 nm. Gel filtration was performed on a Sepharose 6B column (100 × 3.0 cm I.D.) in 0.01 *M* sodium phosphate pH 7.0 containing 0.9% sodium

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chloride at 18 ml/h flow-rate and 6-ml fractions were collected. Purity of ferritin samples was checked by sodium dodecyl sulphate (SDS) polyacrylamide slab gel electrophoresis using the Laemmli method[3]. The running gel contained 15% (w/v) acrylamide, 0.1% (w/v) SDS, 0.375 *M* Tris–HCl pH 8.8 and the stacking gel 4.5% (w/v) acrylamide, 0.1% (w/v) SDS and 0.125 *M* Tris–HCl pH 6.8.

Analytical isoelectric focusing was performed in thin slabs of 6% (w/v) acrylamide containing 2% (w/v) ampholytes in ranges pH 4.0–6.0 and 3.5–10 in a 2:1 (v/v) ratio, by using an LKB 2117 Multiphor apparatus (LKB Instruments, South Croydon, U.K.) at 25-W constant power for 3.5 hr[4]. Gels were stained with Coomassie Brilliant Blue R250.

RESULTS

A typical elution profile for a crude horse liver ferritin sample is displayed in Fig. 1. The reapplication of the ferritin peak (fraction numbers 80–100) to the chromatofocusing column is also shown. On analysis of the horse liver ferritin peak fractions, by SDS–polyacrylamide gel electrophoresis (PAGE), small quantities of impurities were found, even after the second chromatofocusing. However, pure ferritin was obtained from either horse spleen or thalassemic human spleen in one or two applications to the column.

Fig. 2 shows the SDS–PAGE analysis of peak fractions of an initial chromatofocusing of a crude thalassemic human spleen ferritin preparation. Pure ferritin, as seen in fraction 3 in Fig. 2, was also obtained after reapplication of fraction 4 and 5 to the ion exchanger column. Peak fractions from this preparation were subjected to analytical isoelectric focusing and are displayed in Fig. 3. A gradual trend from relatively basic to more acidic isoferritin bands are seen across the ferritin peak as the pH gradient develops (see Discussion).

Gel filtration on Sepharose 6B is often used as a purification step in the preparation of ferritin and is compared here with chromatofocusing. A horse liver ferritin sample was applied to the Sepharose 6B gel filtration column before and after a chromatofocusing step. Fig. 4a shows the chromatofocusing of horse liver ferritin

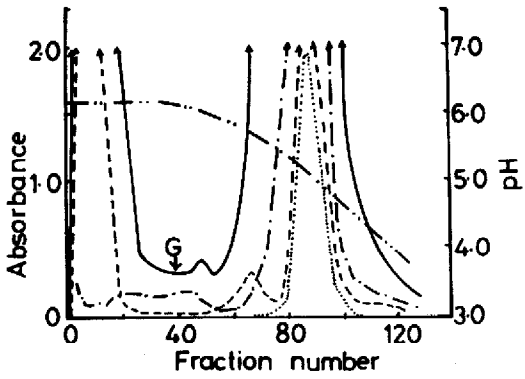

Fig. 1. Elution profile of a crude horse liver ferritin preparation from PBE 94 (25 × 0.9 cm I.D.) monitored at 280 nm (———) and 420 nm (---------). The pH gradient formed (—··—) by the elution of the column with PB 74 pH 4.0 commenced at position G. Fractions 80–100 were pooled, reapplied to the column and monitored at 280 nm (—·—) and 420 nm (·····) as before.

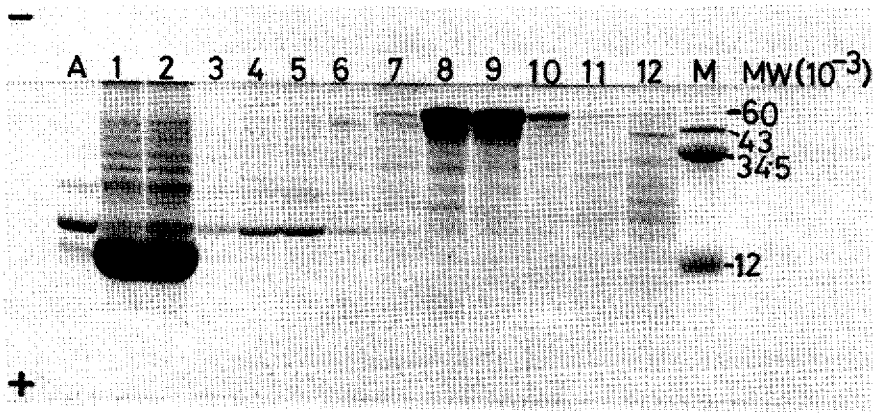

Fig. 2. SDS–PAGE of peak fractions of an initial chromatofocusing of a crude thalassemic human spleen ferritin preparation. A shows a human liver apoferritin standard and M, molecular weight markers. Fractions 1 and 2 eluted from the column before the application of the gradient. Fractions 3–12 contained the main peak eluted from the column during gradient elution.

after gel filtration on Sepharose 6B. Note the large peak eluted before the commencement of the gradient (at point G indicated on the profile). Fig. 4b shows the reverse of the two procedures. SDS–PAGE on peak fractions showed that chromatofocusing alone removed more impurities than gel filtration on Sepharose 6B alone. Chromatofocusing followed by gel filtration gave a cleaner product than the reverse procedure; namely gel filtration followed by chromatofocusing.

DISCUSSION

Our results show chromatofocusing to be a useful technique for rapid processing of crude ferritin samples. Several hundreds of milligrams of protein can be processed in one step and the sample volume applied is unimportant as long as the band of interest has not been eluted before all the sample has been applied.

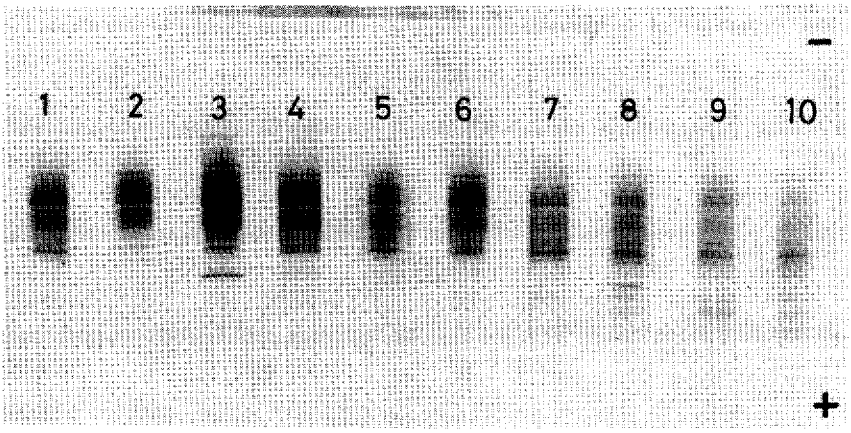

Fig. 3. Analytical isoelectricfocusing of peak ferritin fractions (tracks 2–10) from thalassemic human spleen after a second application to PBE 94. Track 1 contains pure thalassemic human spleen ferritin.

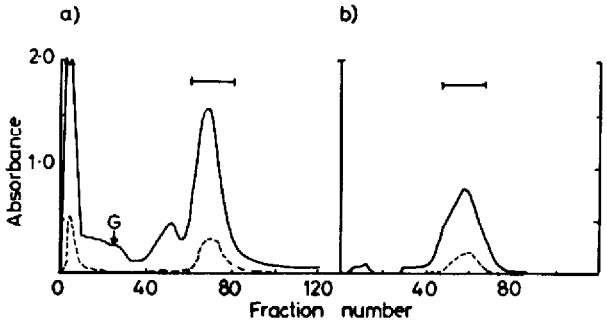

Fig. 4. (a) Elution profile of horse liver ferritin on PBE 94 (25 × 0.9 cm I.D.) after gel filtration on Sepharose 6B (100 × 3.0 cm I.D.) monitored at 280 nm (———) and 420 nm (---------). Elution with Polybuffer 74 pH 4.0 commenced at position G. (b) Elution profile of horse liver ferritin on Sepharose 6B (100 × 3.0 cm I.D.) after chromatofocusing on PBE 94 (25 × 0.9 cm I.D.) monitored at 280 nm (———) and 420 nm (---------).

The pH gradient is automatically formed as the eluting Polybuffer titrates the Polybuffer exchanger, giving a very reproducible gradient and eliminating the need for a gradient maker.

Human and horse spleen ferritins were easily purified by chromatofocusing and were pure after one or two applications to the ion exchanger column. The greater part of impurities present in human and horse liver samples was removed by this technique. Any remaining impurities were removed by a single ultracentrifugation at 100,000 *g* for 2 h.

Chromatofocusing gave a more efficient purification step when compared with gel filtration of Sepharose 6B. Combination of the two procedures showed that an initial chromatofocusing step was preferential. On isoelectric focusing, ferritins from a single source focus over a relatively wide range of isoelectric points beyween pH 4 and 6. They are divided into discrete bands or "isoferritins"[5] showing broad profiles which extend over approximately one pH unit. During this investigation we observed that the isoelectric focusing patterns, from ferritin peak fractions of the chromatofocusing column, showed some fractionation of isoferritins. This indicates the possible application of chromatofocusing to the preparative separation of isoferritins, although this was not investigated in detail in the present study.

ACKNOWLEDGEMENT

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