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Note

Fractionation of human serum albumin isoforms with chromatofocusing

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Chromatofocusing is a relatively new technique for protein separation in which a pH gradient is developed within the column by making use of the buffer capacity of the exchanger [1, 2]. This article describes the application of this technique in the fractionation of albumin

EXPERIMENTAL

Reagents

Polybuffer exchanger PBE 94, polybuffers 96 and 74 and columns were obtained from Pharmacia (Uppsala, Sweden) All other chemicals were of analytical grade

Albumin purification

Serum albumin was purified from normal subjects with pseudo-ligand chromatography on Affi Gel Blue [3] (Bio-Rad, Richmond, CA, U S A.) and analysed by immunoelectrophoresis according to Grabar and Williams [4]

Chromatofocusing

Chromatofocusing was carried out in 20 × 0 9 or 40 × 0 9 cm I D columns according to the manufacturer's instruction manual [5] The polybuffer exchanger was PBE equilibrated with various buffers (Tris–hydrochloric acid, Tris—acetate, Tris—formiate). A mixture of PBE 96 and PBE 74 (25·75—40.60)

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was used for eluting albumin isoforms. The flow-rate was kept constant at 10 ml/h The absorbance of the eluted protein was measured at 280 nm, and recovery was determined using the Coomassie G 250 dye-binding assay [6], the pH gradient was determined at room temperature using an IL 269 pH meter (Instrumentation Lab., Milan, Italy) In all cases, 2 mg of albumin of the purest grade at immunoelectrophoresis were applied to the column

Isoelectric focusing and sodium dodecyl sulphate—polyacrylamide gel electrophoresis (SDS—PAGE)

Ultra-thin isoelectric focusing (240 μm) was carried out in polyacrylamide gels cast on silanized glass plates [7].

SDS—PAGE was performed according to Weber and Osborn [8] and proteins were stained with the photochemical silver method [9]

RESULTS

The albumin fractionating power of PBE 94 is strictly dependent on the type of counter-anion used Fig 1 a—d shows that. (a) when Cl^- is used in both the equilibrating buffer (Tris—hydrochloric acid, pH 8) and in the PBE mixture, albumin remains almost completely bound to the exchanger in spite of the formation of a pH gradient from 8 to 4, the protein emerges from the column with two peaks (the first accounting for ca. 45% of starting material and the other for 50%) when a gradient of sodium chloride from 0 to 1 *M* is applied, (b) the trend is competely reversed if COO^- is used instead of Cl^- in both buffers (Tris—acetate and PBE), albumin elutes now with three peaks, the main of which accounts for ca 80% of the total, (c) when both Cl^- and COO^- are used alternatively in one of the two buffers, a mixed pattern is obtained with three peaks (50% of starting material) eluting at their p*I* values, while two others are retained on the column and emerge with the sodium chloride gradient, (d) finally, albumin is fractionated in four isoforms if the lone gradient of 0—1 *M* sodium chloride is used as eluent instead of the PBE mixtures.

That the peaks emerging from the column are indeed albumin is shown in

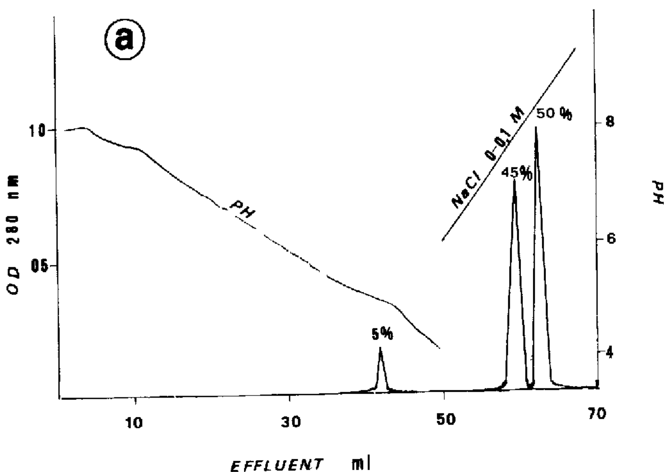

Fig 1

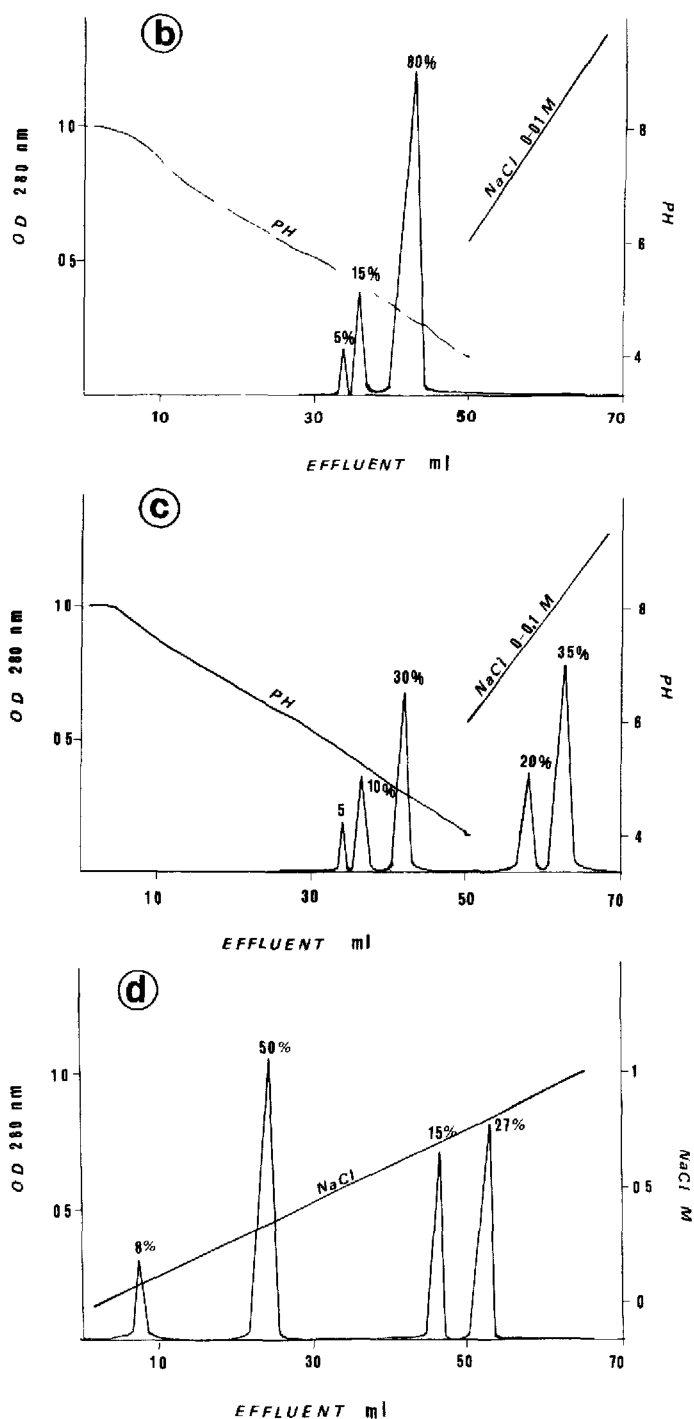

Fig 1 Chromatofocusing of human serum albumin on a 20 × 0 9 cm I D column, working at room temperature with a constant flow-rate of 10 ml/h Conditions of chromatography were (a) equilibrating buffer Tris—hydrochloric acid (pH 8), eluting buffer PBE 96- PBE 74 (25 75) with hydrochloric acid, (b) equilibrating buffer Tris- acetate (pH 8), eluting buffer PBE 96—PBE 74 (25 75) with ethanol, (c) equilibrating buffer as in a, eluting buffer as in b, (d) equilibrating buffer Tris—hydrochloric acid (pH 8), eluting buffer a 0—1 *M* gradient of sodium chloride In all cases, the recovery of protein determined with Coomassie G-250 after dialysis of albumin was ca 100% of the starting material

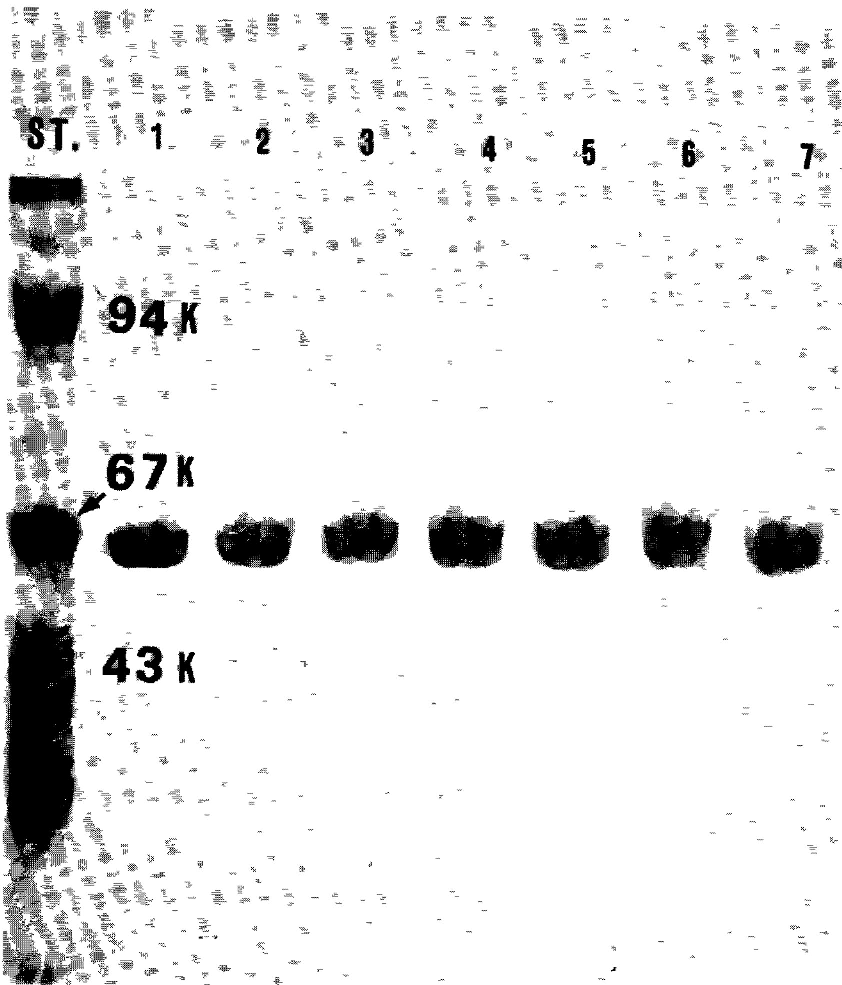

Fig 2 SDS–PAGE of albumin fractions emerging from the PBE with a 0–1 M sodium chloride gradient (experiment of Fig 1 a–d) All fractions were dialysed against water in Thomas membranes (cut-off, 10 000 Dalton)

Fig 2, which is an SDS—PAGE pattern of peaks emerging with sodium chloride in different experiments Furthermore, the same results were obtained when re-chromatographing the dialysed peaks.

As a further probe to evaluate the influence of Cl^- on the interaction between albumin and PBE exchanger, we have performed two experiments in which the amount of equilibrating buffer and the exposure time were varied from a minimum of 20 ml (Tris—hydrochlroic acid, pH 8) for 1 h to a maximum of 300 ml for 10 h Indeed, this manipulation produced a change in the amount of protein tightly bound to resin, which was greater by a factor of 5–6 in the second experiment (large volume, long period) compared with the first one carried out for a short exposure period and with a low volume of buffer

Since one possible explanation for the behaviour of albumin in chromato-

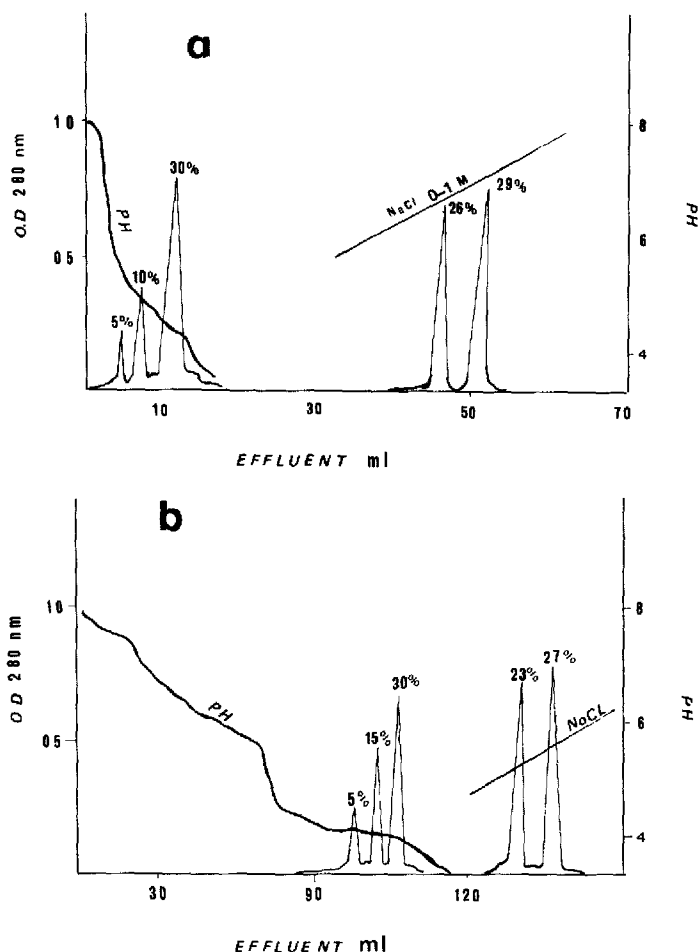

Fig 3 Chromatofocusing of human serum albumin on a 20 × 0 9 cm I D column In all cases, the protein was eluted with a mixture of PBE 96 and PBE 74 (25 75) containing ethanol In experiment a, the equilibrating buffer was 0 005 *M* Tris- hydrochloric acid (pH 8), in b, it was 0 1 *M* Tris—hydrochloric acid (pH 8)

focusing is the hydrophobic interaction of the protein with the resin in the presence of Cl^-, we have repeated the experiment shown in Fig 1c, varying the molarity of the equilibrating buffer (Tris—hydrochloric acid, pH 8) from 0 005 to 0.1 *M* In the first case, we observed that the pH gradient through the column developed much faster than when using the high-molarity buffer (20 vs. 100 ml) (Fig 3); however, in no case was there change of the two fractions retained on the column and eluted with sodium chloride

Finally, we wondered whether a variation of column height or polybuffer dilution would have any influence on albumin separation At least for the amount of albumin used by us (2 mg), no variations of chromatograms were obtained either by increasing the column height from 20 to 40 cm or by varying the dilution of polybuffer from 1 5 to 1 10 (not shown), a 1 7 dilution was therefore chosen as a standard condition

DISCUSSION

These findings demonstrate that the fractionation of albumin by means of chromatofocusing is dependent on the type of counter-anion used when employing COO^-, albumin is fractionated in three isoforms eluting at their p*I* values between 4.6 and 4.9, in the case of Cl^-, only a negligible part of the protein emerges from the column at its p*I* value while the major part is retained by the resin and elutes with sodium chloride. Furthermore, this behaviour is not influenced to any significative extent by the molarity of interacting buffers nor by the height of the column. Finally, when Cl^- is used as counter-anion, the binding properties of PBE exchangers are correlated with the exposure time and with the amount of equilibrating buffer On this basis, it seems reasonable to conclude that, apart from the electrostatic forces, other interactions (mainly hydrophobic) drive the chromatofocusing behaviour of albumin From this point of view, this protein represents a particular case, in that it is widely microheterogeneous with respect to its conformation [10], sulphydryl group content and fatty acid binding, but not with respect to the isoelectric properties, which vary for normal albumin in a narrow p*I* range (4.5–4.8) We conclude that chromatofocusing has unexpected characteristics, which offer some advantages for fractionation of albumin, compared to isoelectric focusing, this technique does not present the side-effects induced by ampholyte binding to the protein It therefore seems conceivable that chromatofocusing may be applied, after adequate standardization, in the molecular characterization of albumin, taking particular care in evaluating the equilibrium existing between isoforms with different p*I* values

This topic is currently under investigation

ACKNOWLEDGEMENT

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