A NEW PROCEDURE FOR THE TWO-DIMENSIONAL DISPLAY OF THE MOLECULAR SIZE-ELECTRIC CHARGE CHARACTERISTICS OF NATIVE PROTEINS IN CRUDE MIXTURES

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1. Introduction

In order to increase resolving power, many workers have combined electrophoretic procedures in two-dimensional techniques for the separation of proteins or protein sub-units. Various combinations of polyacrylamide gel electrophoresis, gel isoelectric focusing, gradient pore electrophoresis, sodium dodecyl sulphate (SDS) gel electrophoresis, gel filtration and immunodiffusion or crossed immuno-electrophoresis have been reported [1–11], but in none of these have the electric charge and molecular size characteristics of native proteins in crude mixtures been clearly and separately displayed. In the present publication this has been achieved by gel isoelectric focusing carried out on proteins already separated by gel filtration.

The new procedure is illustrated by its application both to an artificial mixture of well characterized proteins, and to the preliminary characterization of an exo-β-*N*-acetyl-D-glucosaminidase present in a crude extract from pig epididymis. The procedure is intended not only as a means of displaying important molecular properties of native proteins in crude mixtures, but also as an aid to a new more systematic approach to protein purification [12].

2. Materials and methods

2.1. *Protein mixtures*

The artificial mixture of well-characterized proteins consisted of a solution (1 ml) containing 10 mg horse heart cytochrome *c* (Sigma, London), 10 mg hen ovalbumin (Sigma), 10 mg human transferrin (Mann Biochemicals, New York), 2 mg human IgG myeloma immunoglobulin (collected and isolated in the laboratory at Stanmore), 10 mg bovine liver catalase (Sigma) and 2 mg blue dextran (Pharmacia Ltd., London). The crude protein mixture containing a β-*N*- acetyl-D-glucosaminidase (EC 3.2.1.30) consisted of an extract of pig epididymis [13] which was concentrated to 30 mg protein/ml by vacuum dialysis using 'Visking' tubing.

2.2. *Gel filtration*

The protein mixtures (1 ml) were applied to a 40 cm × 2.5 cm column of Sephadex G 200 superfine (Pharmacis) equilibrated at 4°C with 0.05 M sodium phosphate buffer pH 6.8 containing 0.5 M sodium chloride, and eluted with the same solution at 4 ml/hr using a Mariotte constant head device. The eluant was monitored continuously at 280 nm in the 1 cm flow-cell of an SP 600 spectrophotometer (Pye-Unicam

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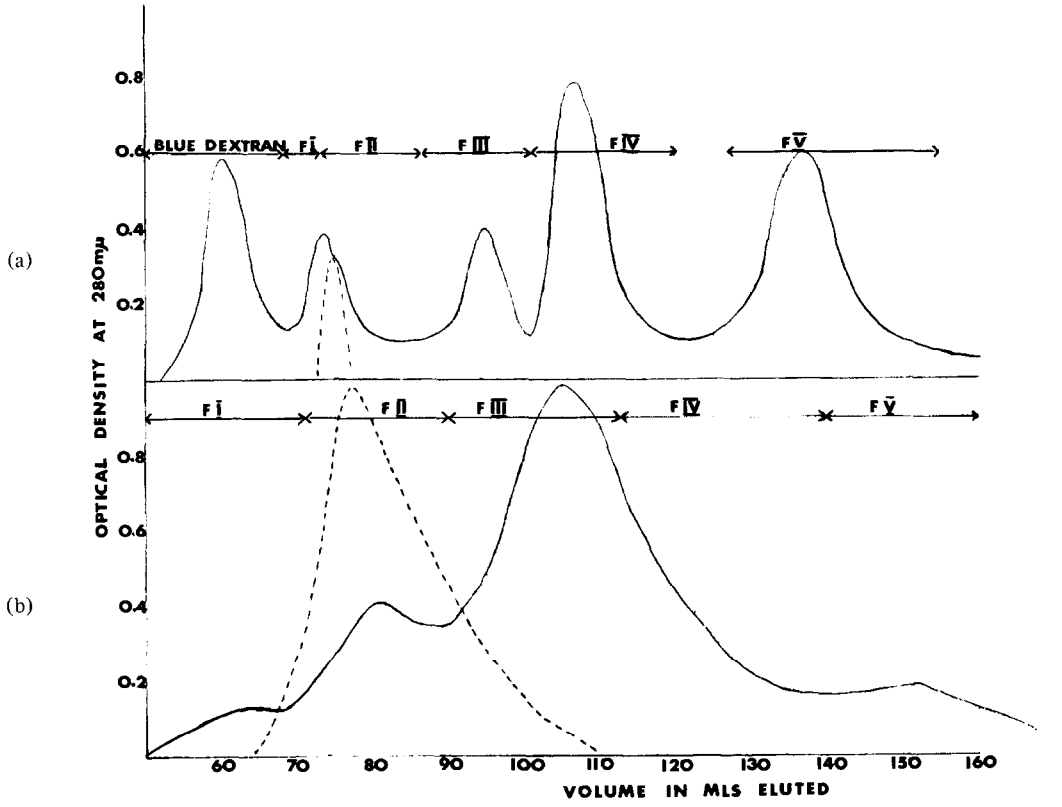

Fig. 1. Elution paterns of protein mixtures from Sephadex G 200 superfine gel filtration. a) Artificial mixture (1 ml) (see Materials and methods). b) Crude vacuum dialysed concentrate (152 mg/ml) of a pig epididymal extract [13]. Elution was with 0.05 M sodium phosphate buffer pH 6.8 containing 0.5 M sodium chloride at 4 ml/hr and fractions (2 ml) were collected and combined. (I–V). Absorption at 280 nm (———). Immunoglobulin and β-*N*-acetyl-D-glucosuminidase activities in (a) and (b) respectively (– – – –).

Ltd., Cambridge, U.K.) and 2 ml fractions collected. Appropriate fractions were combined, concentrated using the Diaflo ultrafiltration membranes (Amicon Ltd., High Wycombe, U.K.) indicated in fig. 2, washed free of salts and, after adjustment to the volume of the original mixture, were subjected to gel isoelectric focusing.

2.3. *Polyacrylamide gel isoelectric focusing*

This was carried out for 20 hr. at 4°C in a pH 3–10 gradient as described by Robinson [14] and the proteins detected with Coomassie Brilliant Blue R 250 (G.T. Gurr, London) at 60°C [15]. β-*N*-acetyl-D-glucosaminidase was located prior to staining for protein by spraying with a fluorogenic substrate [16].

2.4. *Determination of enzyme and protein activities*

IgG immunoglobulin was estimated by radial immunodiffusion on plates supplied by Behringwerke, Marburge-Lahn, Germany. β-*N*-acetyl-D-glucosaminidase was assayed fluorimetrically [16].

3. Results and discussion

When subjected to gel filtration on Sephadex G 200 superfine, the elution profile of the components of the artificial mixture of proteins was as shown in fig. 1a. In fig. 2a, can be seen the first example of the new technique, with the components of the artificial mixture appearing on separate tracks according to whether they fall within certain molecular size ranges,

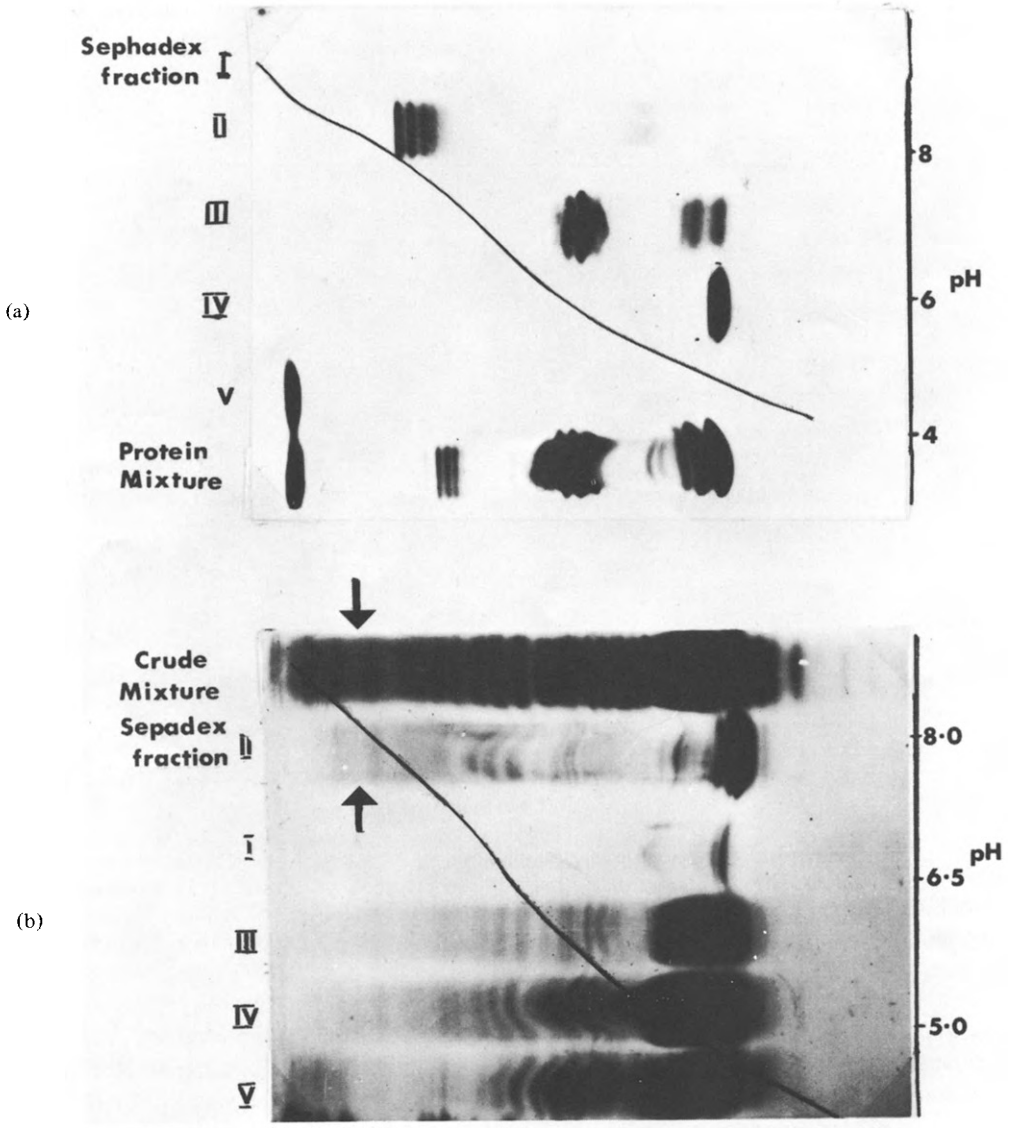

Fig. 2. 'Two-dimensional' molecular size/isoelectric point separation of protein mixtures. a) Combined fractions from the artificial mixture (I–V, fig. 1a) were washed and concentrated by ultrafiltration (I and II-CF50, III and IV-UM10, V-UM2). b) Combined fractions (I–V, fig. 1b) from the epididymal extract were washed and concentrated (I–III-CF50, IV and V-UM10 membranes). Electrofocusing was for 20 hr at 4°C in a pH 3–10 thin-layer polyacrylamide gel [14] and subsequently stained with Coomassie Blue [15]. β-*N*-acetyl glucosaminidase activity located on the gel (→) prior to staining by spraying with a fluorogenic substrate [16]. pH gradient (———).

and from right to left according to the acidity or basicity of their isoelectric points.

The components in the protein mixture were deliberately few in number in order to demonstrate the principles of the technique. While the resolution of the lower molecular weight proteins (cytochrome *c* and ovalbumin) on gel filtration seemed good (fig. 1a), the subsequent isoelectric, focusing stage (fig. 2a) indicated that the resolution of the other proteins was less complete. This relatively low resolving power

of the gel filtration procedure was reason for it being used as the first stage in the new technique and not the second. The elution profile of a crude concentrated extract of pig epididymis is shown in fig. 1b, and the application of the new technique to the preliminary characterization of the β-*N*-acetyl-D-glucosaminidase is illustrated in fig. 2b. It can be seen that the enzyme has a molecular weight in the region of 100000–150000 daltons, and an isoelectric point of about 8.3. Moreover, since the various fractions were applied to the isoelectric focusing plate at concentrations corresponding to those at which they occurred in the original mixture, the technique also gives a visual impression of the purification that has been achieved (about 5-fold) after the gel filtration stage, and also suggests strategy for further purification by methods dependent upon the ionic characteristics of the component proteins.

Isoelectric focusing in slabs of polyacrylamide gels was chosen for the charge characterization of proteins since it is of high resolving power, is applicable to crude protein mixtures, and is ideal for comparative purposes. Of the methods currently available which were considered for the molecular size characterization of proteins, SDS gel electrophoresis was unsuitable since it is applicable only to denatured protein subunits and is difficult to apply to crude mixtures. Gradient pore electrophoresis [17] was not used since it is only applicable to globular proteins of greater than about 50000 daltons [18], and the effects of electric charge differences in the proteins are difficult to eliminate [19]. Thin-layer gel filtration [20] seemed a possibility for the first stage of a two-dimensional technique (its relatively low resolving power makes it undesirable for the second) but it proved difficult to remove the substantial quantity of salt which is necessary for this procedure (but undesirable for isoelectric focusing) and the very small amounts of proteins available from thin layer plates usually made further manipulations impractical.

The possibility of ultrafiltration in stirred cells through successive membranes of decreasing porosity (Amicon Ltd.) was investigated, but in our experience (unpublished results) and that of others [21,22], this is not a reliable or effective method for fractionating crude mixtures of proteins according to molecular size.

Since the separate tracks on the isoelectric focusing plates (figs. 2a and 2b) represent proteins within several ranges of molecular size, the new technique is not truly two-dimensional. It does however have the advantage that the results are directly relatable to methods for the preparative separation of proteins. In this context, the new technique has already proved valuable and effective in arriving systematically at a procedure for the separation of the exo-*N*-acetyl-D-glucosaminidase from pig epididymis [12].

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