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POLYACRYLAMIDE GRADIENT ELECTROPHORESIS FOR PROTEIN PURIFICATION ON THE MILLIGRAM SCALE

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SUMMARY

A preparative-scale electrophoretic technique for protein fractionation and elution on a discontinuous gradient of acrylamide is described, which permits the separation and elution of a pure protein from a mixture containing 4–20 electrophoretically different proteins. The sharpness of the gradient electrophoretic resolution is demonstrated by the separation of proteins consisting of bovine serum albumin polymers and lactate dehydrogenase and enzymes such as acid phosphatase. The compositions of various discontinuous gradients of acrylamide and their application to enzyme purification are discussed. It was found that 60% of the enzyme activity loaded on the gel is recovered after gel fractionation and elution.

INTRODUCTION

Polyacrylamide gel electrophoresis has been widely used for both analytical and preparative purposes[1–30]. The technique described here, involving the use of polyacrylamide gel gradients, was developed in order to purify labile enzyme preparations in view of the shortcomings of classical methods of protein fractionation.

Several types of apparatus for electrophoresis or elution for large-scale preparative gel electrophoresis have been described. Lewis and Clark[1] were the first to adapt the Ornstein[2] and Davis[3] disc electrophoresis method to a preparative scale and to develop a system for collecting the bands of proteins, as they emerged from the end of the column, by a perpendicular flow of buffer. However, the tapered shape of the elution column caused heating during elution. Jovin *et al.*[4] tried to solve this problem by designing an apparatus for preparative, temperature-regulated, polyacrylamide gel electrophoresis, which required a complicated cooling installation.

A review of different elution processes was given by Hjerten *et al.*[30]. Several papers since 1963 have described various types of elution apparatus that involved continuous electrophoresis and collection of the proteins. As they emerged from

the bottom of the gel, the proteins were carried to a fraction collector by one or several perpendicular flows of buffer[4,6,17]. This method has some disadvantages as far as protein recovery is concerned because of dissipation of proteins present in low concentrations by thermal convection, mixing of two bands of proteins, well separated on the acrylamide column, in the eluting chamber owing to the convexity of the bottom of the gel, and dilution of the eluted fraction owing to the continuous flow of eluting buffer.

Other elution systems that have been proposed require the application of electrophoresis following homogenization of gel sections[11] or electrodialysis of excised pieces of gel[12]. The excision process used by Lewis and Clark[1], involving homogenization in buffer and centrifugation of the pieces of gel, has the disadvantage of a large recovery volume.

With these considerations in mind, we attempted to devise a simple technique for preparative polyacrylamide gel electrophoresis, with an elution system that would minimize the inactivation of unstable enzymes and ensure the recovery of proteins in concentrated solutions with a good yield. These aims were achieved by the introduction of a discontinuous gradient of acrylamide, whose resolution greatly exceeds that observed with the classical acrylamide technique, and an electrophoretic elution of the gel sections in 1-ml collecting tubes.

MATERIALS AND METHODS

Chemicals

Acrylamide, N,N'-methylenebisacrylamide, β-diethylaminopropionitrile, potassium hexacyanoferrate(III), ammonium persulphate, Tris and glycine were purchased from Merck (Darmstadt, G.F.R.). Coomassie Brilliant Blue R 250 and bovine serum albumin (BSA) were obtained from Mann Labs. (New York, N.Y., U.S.A.), trypsin from Armour Labs. (London, Great Britain) and pepsin and phosphorylase from Worthington (Freehold, N.J., U.S.A.). Amido-Schwartz was purchased from Apelab (Bagneux, France), lyophilized acid phosphatase, grade II, from potato from Boehringer (Mannheim, G.F.R.) and lactate dehydrogenase (LDH), band 1 native (4H), from pig heart from Boehringer (ref. 15378).

Phosphatase assay

The reaction mixture contained (in a total volume of 1.1 ml) 1 μmole of *p*-nitrophenyl disodium phosphate, 150 μmole of acetate buffer (pH 5.0), 10 μmole of EDTA and the enzyme solution. After 10 min of incubation at 37°, the reaction was stopped by adding 0.2 ml of 2 *N* aqueous ammonia. The absorption at 400 nm was measured against a suitable blank.

Protein determination

Protein was measured using a micro-biuret technique[14].

Electrophoresis apparatus

The apparatus (Fig. 1) was constructed on the same principle as the commercially available analytical model from Pleuger (S. A. Wijnegem, Belgium) and adapted to give a large-scale model. It is made from a Plexiglass cylinder 14 cm in diameter and

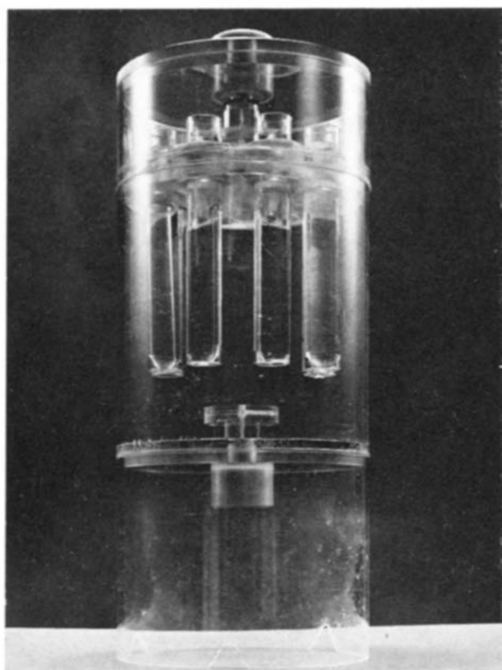

Fig. 1. Electrophoresis apparatus.

includes two tanks, the upper tank, 6.4 cm high, having eight holes of I.D. 30 mm arranged at 45° intervals around the centre. Each hole is fitted with a plastic screw system (Fig. 2) which allows the glass columns, previously filled with polyacrylamide gel, to fit in easily and safely. The lower tank, having the same diameter, is 15 cm high. One of the two circular Plexiglass electrodes is fixed in the lower chamber while the other is part of the apparatus cover.

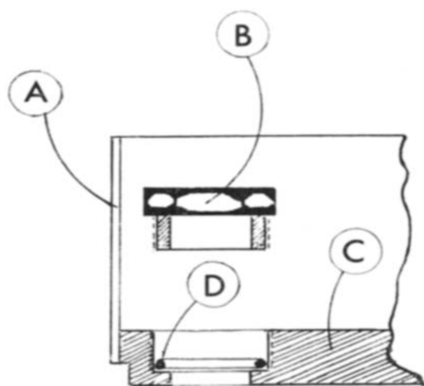

Fig. 2. Cross-section of the system for fixing the electrophoresis glass column in the upper tank (A). B, Plastic ring; C, bottom of the upper tank of the electrophoresis apparatus; D, O-ring.

Gel slicer

A Plexiglass gel slicer (Fig. 3) was adapted from the model described by Lewis and Clark[1]. It has 0.2-cm spaced slots and a central hole of 2.0 cm diameter, allowing the gel to be held in position while being sectioned with a steel wire.

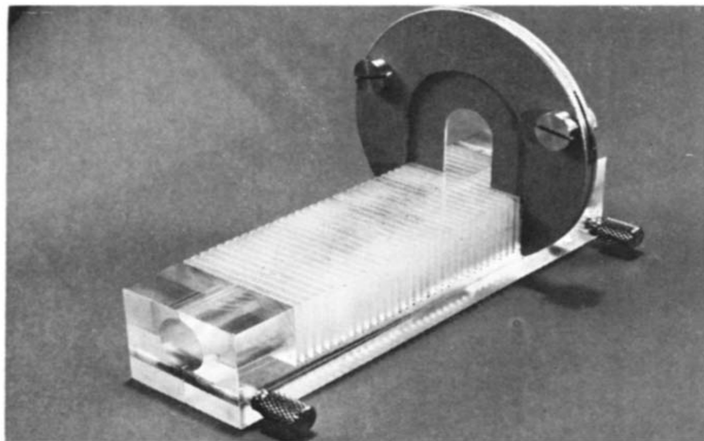

Fig. 3. Gel slicer.

Elution apparatus

The system (Fig. 4) is made of a wide Plexiglass cylinder 29 cm in diameter and 40 cm high. It is composed of two sections placed one on the top of the other, the upper one having eight holes to allow the insertion (Fig. 5, I) of the elution columns (Fig. 5, F) of 5.2 cm diameter, in which squashed gel sections are placed.

The columns are closed at the top end with a nylon diaphragm and at the bottom end with a glass-wool plug (Fig. 5, G and H). At the bottom of the elution column (Fig. 5, L) collecting tubes are fitted (1-ml volume) which are closed off with a dialysis membrane (Fig. 4) held in place by a rubber-band. The collecting tubes are immersed in the buffer contained in the lower section to which one electrode is attached, the upper electrode being part of the apparatus cover.

Preparation of reagents

The following were prepared.

(1) A solution of acrylamide (40 g per 100 ml) and bisacrylamide (1.06 g per 100 ml) was used for forming gels containing acrylamide in the range 3–10%. This solution, after appropriate dilution, was mixed with an equal volume of reagents (2), (3) and (4) described below.

(2) A 1.6-g amount of β-diethylaminopropionitrile was dissolved in the Tris–glycine stock buffer diluted 2.5 times (30.3 g/l of Tris and 144 g/l of glycine).

(3) Potassium hexacyanoferrate(III) solution, 0.03%.

(4) Ammonium persulphate solution, 0.48%.

(5) The electrophoresis buffer consisted of 25 m*M* Tris–glycine (pH 8.3), prepared by dilution of 0.25 *M* Tris–glycine concentrate (30.3 g/l of Tris and 144 g/l of glycine).

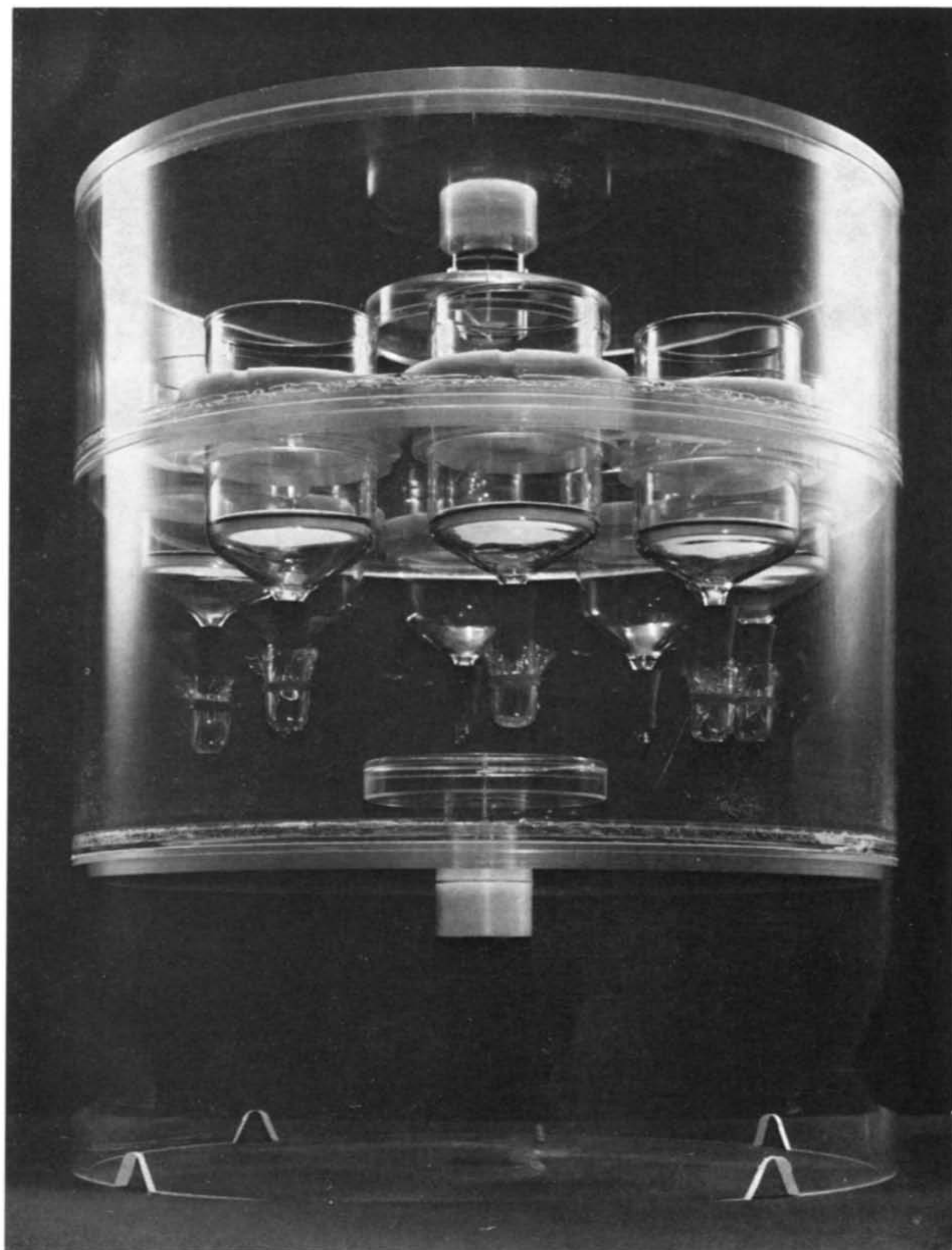

Fig. 4. Elution apparatus.

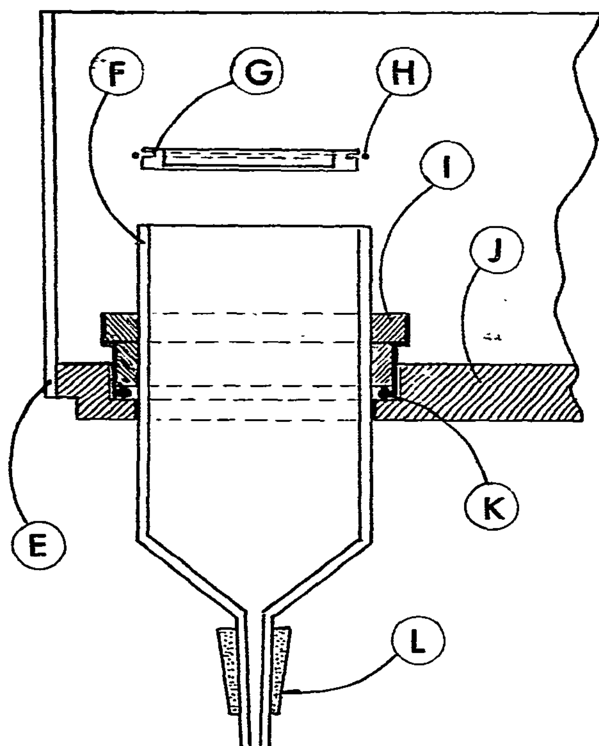

Fig. 5. Cross-section of an elution glass vessel (F). J, Plastic bottom of the upper tank (E) of the elution apparatus; I, plastic screw; K, O-ring; G, nylon cover tightened by a plastic ring (H).

(6) Amido-Schwartz stain: 50 mg of Amido-Schwartz dissolved in a mixture of 45 ml of methanol, 45 ml of water and 10 ml acetic acid.

(7) Coomassie Blue stain: 0.25 g of Coomassie Brilliant Blue R 250 dissolved in a mixture of 45 ml of methanol, 45 ml of water and 10 ml of acetic acid.

(8) De-staining solvent: 200 ml of methanol plus 70 ml acetic acid diluted to 1000 ml with distilled water.

Preparation of gels

The gels were formed by mixing the appropriate reagents in solution and allowing them to polymerize directly in the tubes (15 × 1.6 cm) in which the electrophoresis was to be carried out. In order to obtain a flat surface the gel was prepared "downwards", with a rubber stopper inserted in the glass tube. Thus, the gradient of acrylamide was set as follows: 5 ml of 5.5% acrylamide solution was first poured and allowed to set until polymerization occurred (about 20 min), the buffer remaining above the gel layer was removed and then 5 ml of 6.5% acrylamide was poured; when this layer had polymerized, 7.5, 8, 8.5 and 9% acrylamide solutions were poured in the same way.

Electrophoresis

In this procedure, designed for thermolabile enzymatic proteins, electrophoresis

was performed at 4° with a constant voltage of 80 V. No heating of the gel occurred under those conditions. The duration of electrophoresis depended on the gradient gel system chosen and the protein material used; it may vary from 4 to 16 h. After electrophoresis, the gels were removed from the glass tubes by directing a fine jet of buffer between the tube and the gel and were stained either in Amido-Schwartz for 30 min or Coomassie Blue for 4 h and then de-stained with solution (8) at 37°. Gels to be eluted were not stained but were cut into slices 0.2 cm thick, the slices being pulped in a syringe and injected with electrophoresis buffer into the eluting tubes (Fig. 5). A current of potential 200 V (≡ 1 mA per column) was passed through the apparatus for 5–10 h and the proteins eluted were recovered in the lower part of the elution system closed with a dialysis membrane. In some instances the protein became partly adsorbed on to the dialysis membrane, but reversal of the current for 3 min served to return it into solution in the eluting buffer.

Loading of protein

Protein samples were made denser than the overlying buffer by addition of an inert solute (10% glycerol or sucrose) before being layered into the gel. About 5–10 mg of protein were loaded per gel.

RESULTS

BSA polymers and LDH isozymes were subjected to different experiments in which either the composition of the polyacrylamide gradient or the time of electrophoresis was changed.

Fig. 6 shows a 7.5% polyacrylamide gel reference (A) and three gradients (B–D). B corresponds to a gradient of 5.5–8.5% polyacrylamide while C and D are identical gradients of 8.5–5.5% polyacrylamide with different electrophoresis times. The four gels were 15 cm in length and 1.6 cm in diameter.

For the gels A, B and C, the electrophoresis was carried out at 4° and 80 V until the bromophenol blue arrived at the bottom of the gels (usually 5 h), while electrophoresis in gel D was maintained for a further 6 h. As shown in Table I, where migration values of BSA monomer, dimer and trimer and LDH are reported, the best results were obtained with polyacrylamide gel of type D.

The measured distances between monomer and dimer protein bands, which are approximately the same in gels A, B and C (16, 13.5 and 17 mm), are more than twice as large in gel D (40 mm). This result also applies to the observed distance in gel D between monomer and trimer (57 mm), monomer and LDH (34 mm) and monomer and tetramer (70 mm).

To test the possible range of the polyacrylamide gradient technique for protein separation the LDH band, which migrates very close to the BSA dimer, was examined using these different gel gradients. A comparison of their migrations in the four gels demonstrates an improvement in their separation: in A 2 mm, in B 3 mm, in C 3.5 mm and in D 6 mm. It should be noted that an increase in the time of electrophoresis increases the separation of the proteins only with polyacrylamide gradient type D; when the time of electrophoresis is increased when using 7.5% polyacrylamide (gel type A), diffusion and mixing of the protein bands occur.

An impure sample of acid phosphatase from potato was chosen in order to

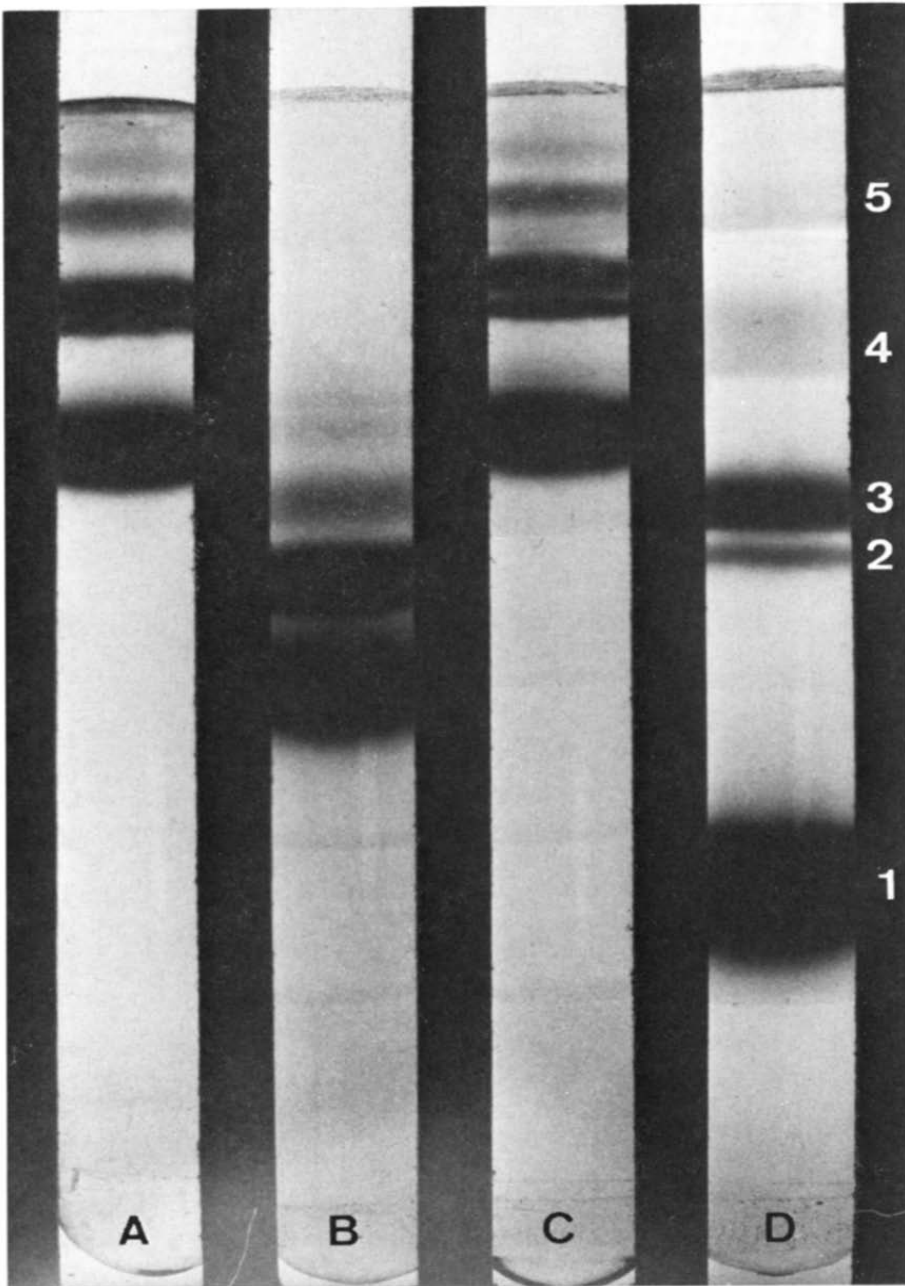

Fig. 6. Separation of BSA polymers and LDH on three different polyacrylamide gradients: A, 7.5% polyacrylamide gel control; B, gradient of 5.5–8.5% polyacrylamide; C and D, gradients of 8.5–5.5% polyacrylamide. 1 = BSA monomer; 2 = LDH; 3 = BSA dimer; 4 = BSA trimer; 5 = BSA tetramer.

TABLE I

MIGRATION OF PROTEINS ON DIFFERENT POLYACRYLAMIDE GELS

The values given represent the migrations in mm. A, B, C and D correspond to the gel shown in Fig. 6.

Protein	*Polyacrylamide gel*			
	A	*B*	*C*	*D*
BSA monomer	36	62	36	83
LDH	22	51.5	22.5	49
BSA dimer	20	48.5	19	43
BSA trimer	11	42	11	26
BSA tetramer	6	33	6	13

develop this method for enzyme separation, purification and elution. The enzyme preparation (acid phosphatase grade II) was examined on three types of gel: a 7.5% polyacrylamide gel (type A), a 5.5–8.5% gradient (type B) and an 8.5–5.5% gradient (type C). Its activity and the corresponding stained protein appeared as a wide and diffused zone in the 7.5% gel (A) containing contaminating proteins, while phosphatase activity was found in a narrower fraction in the two polyacrylamide gradients distinctly separated from the contaminants. It was also found that the acid phosphatase did not migrate further than the 6.5% acrylamide stage in gradient B. Therefore, as shown in Fig. 7, we reduced the number of polyacrylamide stages to four, *viz.*, 5.5, 6, 6.5 and 7%, and also doubled their length. Unlike LDH and BSA polymers, the phosphatase separation was better in gel gradient type B than type C.

As shown in Fig. 8, by using this procedure the phosphatase was separated and eluted as one pure protein.

To study the recovery of proteins, trypsin, pepsin, lactate dehydrogenase, phosphorylase a and purified acid phosphatase were submitted to a type B gradient and a standard 7.5% polyacrylamide gel. The recoveries from these two types of gel were similar, showing that the proteins were delayed at each surface in the polyacrylamide gradient but were not adsorbed on to these surfaces.

Table II shows the proportions of protein recovered and the recovery of phosphatase activity in the elution system described above. The proportion of protein recovery varied between 60 and 80%. A recovery of 60% was obtained for the acid phosphatase activity.

TABLE II

PROTEIN RECOVERY

Protein	*Molecular weight*	*Protein (% eluted)*	*Enzyme activity (% eluted)*
Trypsin	23,000	80	—
Pepsin	35,000	80	—
LDH	140,000	60	—
Phosphorylase *a*	94,000	70	—
Acid phosphatase	—	—	60

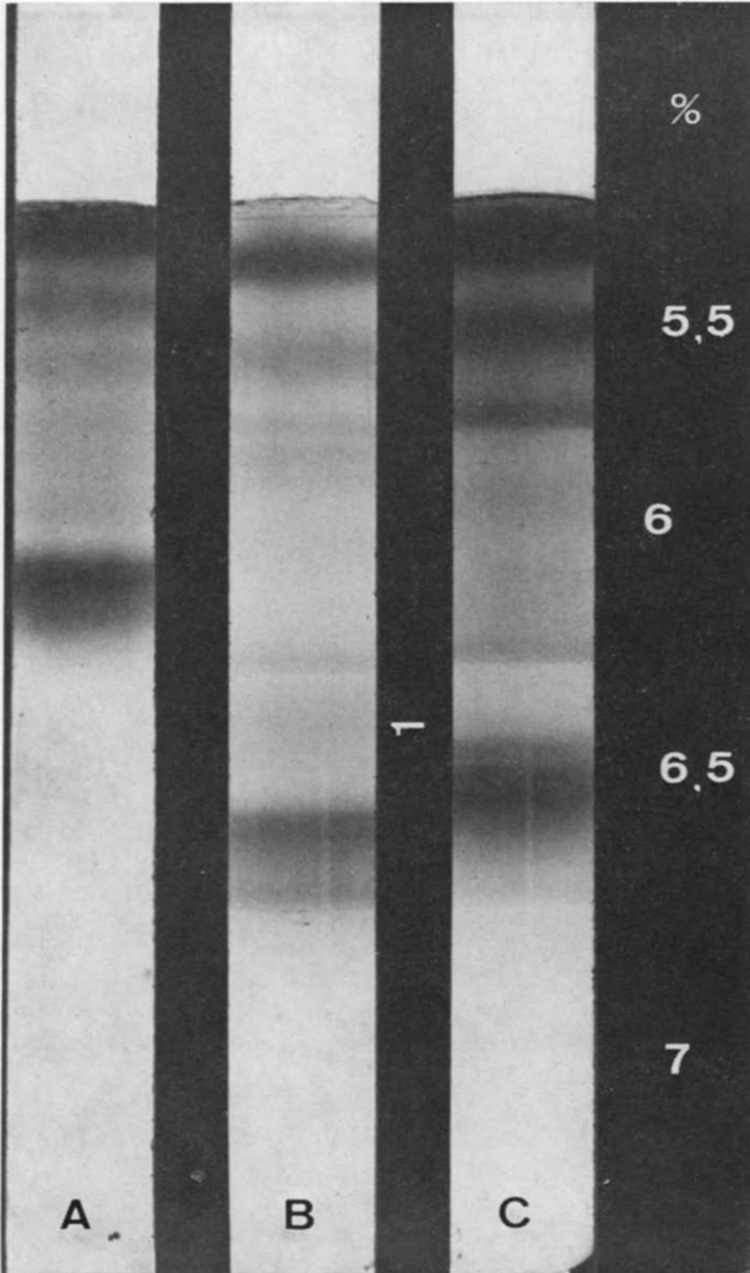

Fig. 7. Separation of acid phosphatase from potato on polyacrylamide gels: A, 7.5% polyacrylamide gel; B, polyacrylamide gradient of 5.5–7%; C, gradient of 7–5.5% polyacrylamide. The arrow shows the position of the acid phosphatase protein band.

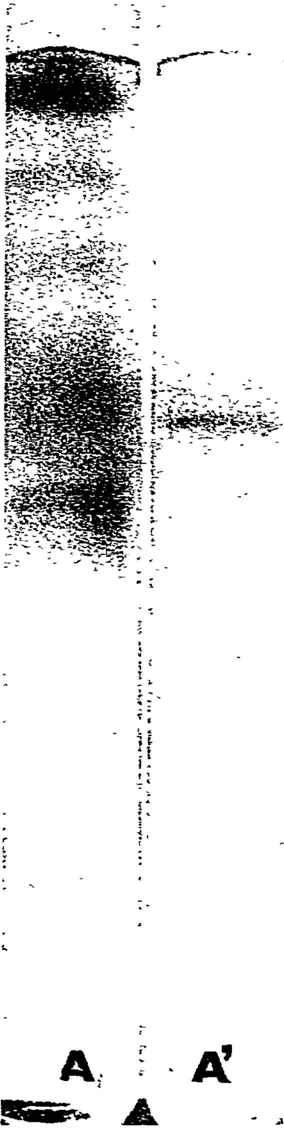

Fig. 8. Disc electrophoresis of the acid phosphatase on 7.5% polyacrylamide gels: A, before separation on polyacrylamide gradient (type B); A′, after elution of a 5.5–7% polyacrylamide gradient (type B).

DISCUSSION

In order to enhance the resolution in polyacrylamide gel electrophoresis, the use of techniques such as pH gradients[19], isoelectric focusing[22,33,34] and linear pore gradient electrophoresis[23,25] for analytical purposes has been proposed. Better resolution was observed with electrophoresis on polyacrylamide gel in a continuous molecular sieve

gradient than on homogeneous gel[26]. This result was confirmed and even improved with the introduction of differential disc electrophoresis for the qualitative separation of serum proteins[20,24] and preparative enzymatic protein fractionation as described here. The most important aspect of this discontinuous polyacrylamide gradient technique is the number and length of the different acrylamide concentration stages.

A greater use of molecular sieving is obtained with electrophoresis through gels with different pore sizes; gels with increasing (from 9 to 5%) or decreasing (from 5 to 9%) pore size permitted the separation of large molecules from the smaller components and the separation of molecules with similar dimensions but slightly different charges.

Low recoveries of proteins in preparative polyacrylamide gel electrophoresis may be related to adsorption of the proteins on the membrane of the elution chamber or during concentration of the too dilute eluate (when continuous elution is carried out)[21]. In the present system, elution in a small volume at 4° prevents dilution and denaturation of the purified enzymatic proteins. Moreover, this method requires simple and inexpensive apparatus that can be constructed in most laboratories.

The reproducibility and reliability of this protein fractionation and elution method, the amount of protein that could be applied on the gels (5 mg/cm^2) and the percentage of protein and enzymatic activity recovered (Table II) demonstrated that this method can be used as a purification step, with a comparable or even better recovery than that obtained in column chromatography. It has been used successfully for the purification of an ATP-dependent deoxyribonuclease from *Bacillus subtilis*[31], a trehalase from pig kidney[32] and glycosidases from alfalfa seeds[18].

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