Reprinted from

Biochimica et Biophysica Acta, 386 (1975) 181–195

BBA 36932

ISOELECTRIC FOCUSING IN LAYERS OF GRANULATED GELS

II. PREPARATIVE ISOELECTRIC FOCUSING

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Received August 5th, 1974)

SUMMARY

A method for preparative isoelectric focusing of 0.1–10 g amounts of proteins is described. For anticonvective stabilization of the pH gradient, layers of granulated gels (e.g. Sephadex or Bio-Gel) of variable length, width and thickness were used either on glass plates or in troughs. Load capacity, defined as the amount of protein per ml gel suspension, was determined to be 5–10 mg per ml for total protein, irrespective of the pH range of the carrier ampholytes. For single proteins load capacities of 0.25–1 mg per ml were found for pH 3–10 carrier ampholytes, and 2–4 mg per ml for narrow pH range ampholytes. Experiments on a quartz plate followed by densitometric evaluation in situ at 280 nm have demonstrated that it is possible to proceed from analytical thin-layer isoelectric focusing to preparative separations without loss of resolution, just by changing the dimension of the gel layer and increasing the protein load. Improved resolution which facilitates isolation of isoelectrically homogeneous components could be achieved on a 40 cm long separation distance. The geometry of a layer is favourable to heat dissipation and thus permits the use of high voltage gradients. Recovery of the focused proteins is high and elution simple. The efficiency of the method is illustrated by examples showing separations of single proteins and protein mixtures.

INTRODUCTION

In recent years many efforts have been directed at the development of efficient analytical techniques of isoelectric focusing [1, 2]. By contrast, preparative isoelectric focusing remained a comparatively neglected field of activity. Different approaches have been suggested for preparative isoelectric focusing including vertical columns with density gradient stabilization [3–5], continuously polymerized (compact) polyacrylamide gels in the form of either flat-bed [6] or cylindrical gels [7], layers of granulated gels [8, 9], zone convection focusing [10], a polyethylene tubing [11],

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multimembrane devices [12], and continuous flow systems with gel or density gradient stabilization [13]. Despite this diversity of experimental approaches few preparative applications have been described, representing only a small fraction of the rapidly expanding literature on isoelectric focusing. Preparative isoelectric focusing in layers of granulated gels is described in detail in the present paper with special emphasis on load capacity and scaling up of the method for fractionation of protein amounts in the range of 0.1–10 g. Preliminary reports have already been presented elsewhere [14, 15].

MATERIALS AND METHODS

Carrier ampholytes "Ampholine" were from LKB Produkter AB, Bromma, Sweden, Sephadex gels, all "Superfine", from Pharmacia, Uppsala, Sweden, and the Bio-Gels (−400 mesh) from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Cytochrome *c*, horse and sperm whale myoglobin, β-lactoglobulin, ovalbumin, ribonuclease, dimethylcasein and the dyes were from Serva, Heidelberg, W. Germany, Pronase E came from E. Merck, Darmstadt, W. Germany.

Apparatus

The "Double Chamber" (Desaga, Heidelberg, W. Germany) has been used both for preparative and analytical isoelectric focusing [16]. Cooling water at 2–10 °C was circulated through the apparatus from a Lauda K2/RD refrigerated constant temperature circulator (Dr Wobser, Lauda, W. Germany). A Type 641 digital pH meter (Knick, Berlin, W. Germany) and a micro flat-membrane glass electrode of approx. 1.5 mm diameter combined with a reference electrode, mounted in a special holder (Desaga, Heidelberg, W. Germany) were used for the pH measurements. Densitometric evaluation was carried out with the Schoeffel Spectrodensitometer SD 3000 (Schoeffel Instrument Corporation, Westwood, N. J., U.S.A.).

Small scale preparative isoelectric focusing

Glass plates 20 × 10 cm, 20 × 20 cm and 40 × 20 cm or a 20 × 10 × 0.4 cm quartz plate (Westdeutsche Quarzschmelze GmbH, Geesthacht, W. Germany) were coated with a thick suspension containing 1% w/v of carrier ampholytes (Fig. 1). The gel suspension was spread on the glass plate by means of a glass rod [16]. The gel was dried in air until a layer was obtained which did not move when the plate was inclined at an angle of 45°. The sample was applied as a streak directly on the surface of gel layer by means of a sample applicator (Desaga, Heidelberg) or simply with a microscopic slide or a rectangular glass plate of a width 2 cm smaller than that of the gel layer (to compensate for any possible edge effects). With larger sample volumes the sample was mixed with dry gel (e.g. approx. 60 mg Sephadex G-75 or approx. 30 mg Sephadex G-200 per ml) and the resulting fairly liquid suspension was poured into a slot. With either method the sample could be applied at any position on the layer, application in the middle of the plate or at halfway towards the anode being preferred in most experiments. Voltage was applied either through platinum band electrodes (Desaga, Heidelberg) or through flat carbon electrodes (Ringsdorff, Bad Godesberg, W. Germany), which were in contact with the gel layer through pads of MN 866 paper (Macherey, Nagel and Co, Düren, W. Germany)

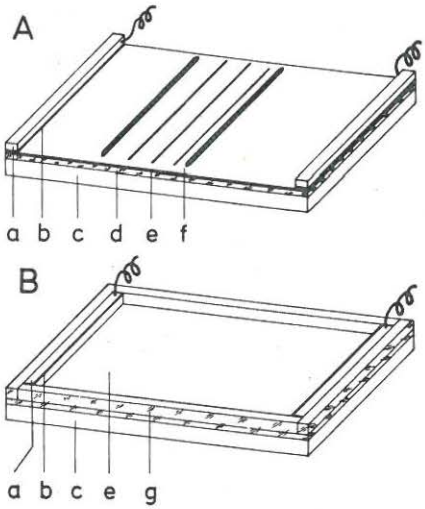

Fig. 1. Preparative isoelectric focusing. (A) small scale separations. (B) large scale separations. a, electrode; b, filter paper pad soaked with electrode solution; c, cooling block; d, glass plate; e, gel layer; f, focused proteins; g, trough.

soaked with the corresponding electrode solutions, 1 M sulfuric acid at the anode and 2 M ethylene diamine at the cathode.

Large scale preparative isoelectric focusing

Troughs, 40 × 20 cm or 20 × 20 cm, consisting of a glass plate at the bottom and a 1 × 1 cm plastic frame (Desaga, Heidelberg) were used with gel layers up to 1 cm thickness (Fig. 1). The gel layer was obtained by pouring the required amount of gel suspension into the trough followed by drying as described for small scale preparative focusing. The sample was applied in the form of a fairly liquid gel suspension into a slot at any desired position of the layer. The platinum band or flat carbon electrodes were inserted vertically into the gel layer resulting in a homogeneous electric field during focusing. Pads of MN 866 filter paper soaked with the electrode solutions were put between the gel layer and the electrodes. The pads and the electrodes were placed into the predried gel layer after removing small amounts of gel at both ends. A small amount of a fairly liquid gel suspension was poured between the paper pad and the gel layer in order to establish good contact. The total gel volumes used in the troughs ranged from 300 to 800 ml.

Detection of proteins

On the quartz plate the proteins were detected directly in the gel layer by densitometry in transmission at 270–280 nm. In all experiments at high load capacities the major components could be seen as transparent zones in the gel layer. For location of the minor components the print technique has been employed [16]. Due to the increased thickness of the gel layer in preparative runs, a thick filter paper e.g. MN 827 was preferred; with thin filter papers variable amounts of gel adhered to the print. The prints were stained with dyes of low sensitivity e.g. Light Green SF

or Coomassie Violet R-150 and evaluated densitometrically in remission. Rapid localization of the proteins by the print technique could be achieved by shortening the staining time and destaining with solutions of higher alcohol content.

Elution

After location of the proteins by direct densitometry, visual inspection or staining the print, the fractions of interest were removed from the gel layer with a spatula. The removed gel was liquefied by addition of a small amount of distilled water and transferred to small glass columns of variable size with a cotton plug at the bottom. The proteins were eluted with water (or buffer in case of precipitated proteins), using a water/gel ratio of 1.5–2.0, by vol. With small gel volumes elution was complete within a few minutes. Carrier ampholytes were removed from the eluate by dialysis or gel chromatography [4].

Other methods

pH measurements were made at 25 °C as described previously for thin-layer isoelectric focusing [16]. Protease activity in the eluates was determined by the dimethylcasein method [17].

RESULTS

Comparison of different gels

Previous experiments have shown that Sephadex G-75 Superfine is the most suitable supporting gel for thin-layer isoelectric focusing [16]. Sephadex gels with higher G-numbers have given satisfactory separations but could not be used in analytical work due to difficulties in obtaining the print. The suitability of different gels, all of the Superfine grade, for preparative isoelectric focusing was examined since the indicated limitation encountered in analytical work seemed to be irrevelant in considering preparative applications. With sperm whale myoglobin, a strongly coloured protein, visual inspection during the course of the experiment was possible. In Fig. 2 the results for three Sephadex gels and a polyacrylamide gel of the Bio-Gel series are shown. In all gels an excellent separation of myoglobin into a total of 10–15 components was achieved. Basically the same results were obtained when instead of focusing at 300 V for 15–16 h followed by 600–800 V for 2–4 h, the focusing time at 300 V was reduced to only 4 h followed by 600–800 V for 4 h which permitted completion of he experiment within 8 h. Much sharper zones were repeatedly observed for the Sephadex gels than for the Bio-Gel series (P-60, P-150, P-200 and P-300). With increasing G-number of Sephadex, total spreading, that is the distance between the most acidic and basic components of myoglobin, progressively increased. For Sephadex G-200 the most shallow pH gradient and correspondingly the greatest spreading and best separation of the individual components was obtained. For the Bio-Gel series total spreading and steepness of the pH gradient did not depend on the P-number up to P-200, and even decreased for P-300. Steepness of the pH gradient and total spreading probably reflect different electrochemical properties of the examined gels. The variation in carboxyl group contents between different Sephadex G-types and different batches of a single type is claimed to be not very large (Fischer, L., Pharmacia, Uppsala, personal communication). Since only half of the dry gel is used for

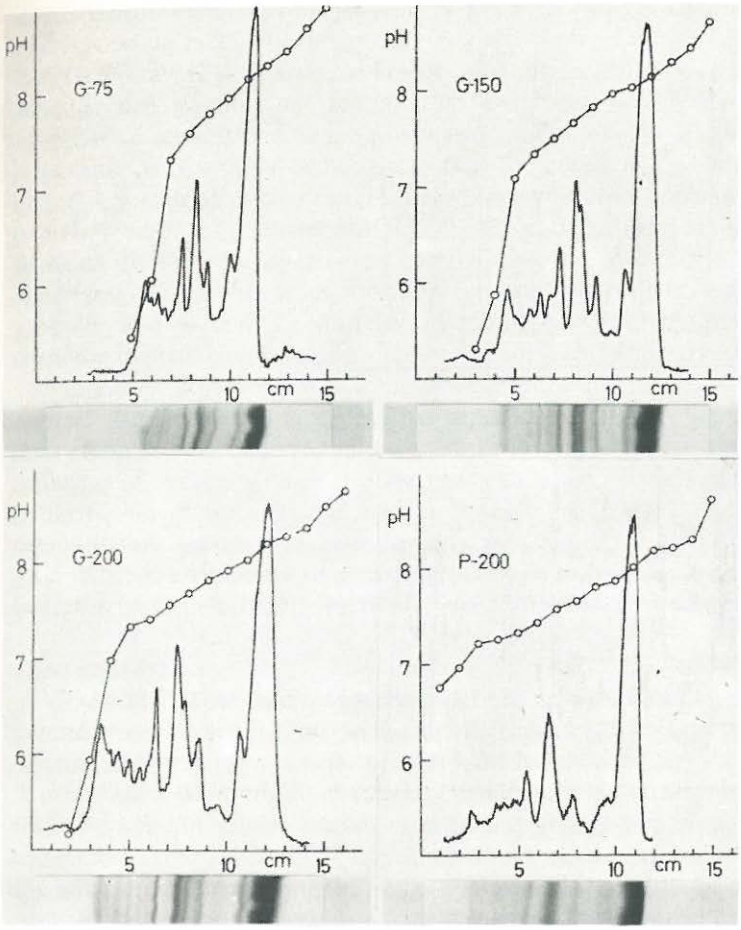

Fig. 2. Preparative isoelectric focusing of 100 mg sperm whale myoglobin in different gels. Plates 20 × 10 cm coated with 50 ml of a gel suspension of Sephadex G-75, G-150, G-200 or Bio-Gel P-200, containing pH 7–9 carrier ampholytes. Focusing: 300 V for 16 h followed by 600–800 V for 2–4 h. Densitograms of the unstained prints shown for each gel at the bottom. ○—○, pH gradient.

preparing a Sephadex G-200 suspension, the resulting reduced contents of charged groups per unit of gel volume could well account for the difference between the Sephadex G-75 and the G-200 gels, an interpretation further substantiated by previous experiments with Sephadex treated with propylene oxide [16].

Focusing on a quartz plate

A number of preparative experiments have been performed with a mixture of proteins consisting of ovalbumin, β-lactoglobulin, myoglobin, ribonuclease and cytochrome *c*. Most of these proteins have been already studied by thin-layer isoelectric focusing and are routinely used as pH markers in this laboratory, thus facilitating comparison of preparative separations with analytical runs [16]. The proportion of these proteins in the mixture was varied to compensate for differences in their extinction coefficients and banding after focusing. Use of a quartz plate permitted

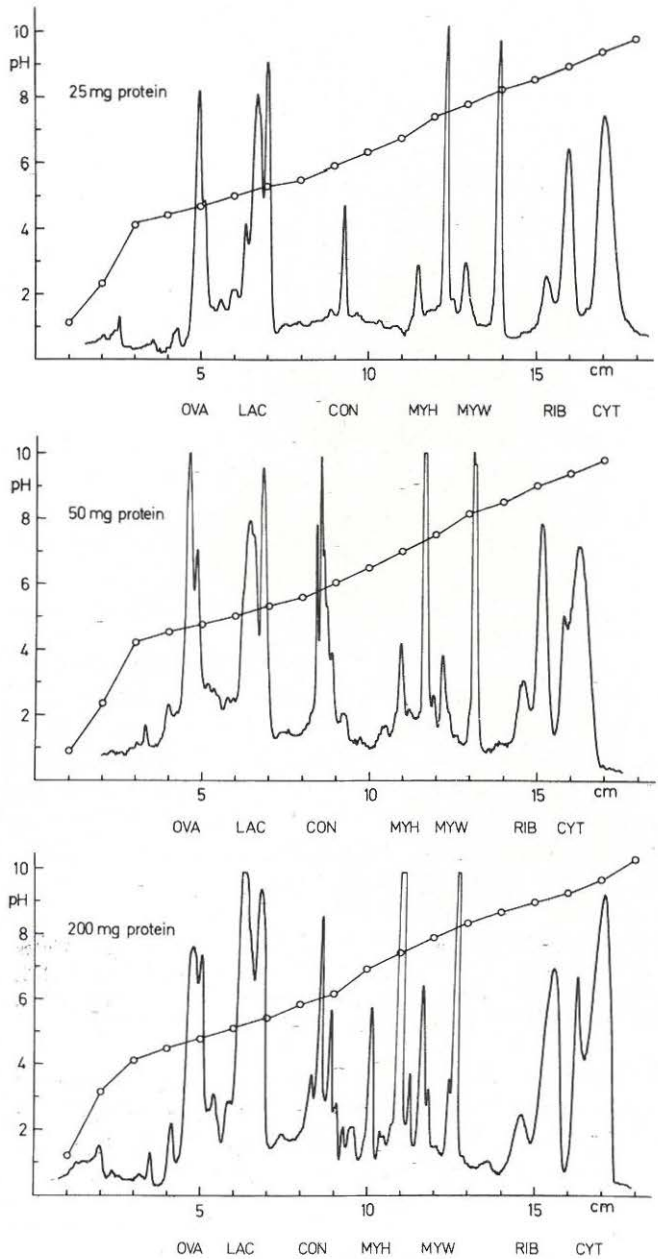

Fig. 3. Focusing of a protein mixture on a 20 × 10 cm quartz plate. Sephadex G-200, pH 3–10 carrier ampholytes. Thickness of the gel layer, 1 mm for 25 mg and 2 mm for 50 mg and 200 mg total protein applied. Sample application in the middle of the plate. Focusing: 200 V for 16 h followed by 600–800 V for 2–4 h. Densitometry in transmission at 280 nm in the gel layer. ○—○, pH gradient. Proteins (mg of each/ml); (OVA), ovalbumin (50 mg, 1 × crystallized) containing as impurity (CON) conalbumin, (LAC) β-lactoglobulin (50 mg), (MYH) horse myoglobin (20 mg), (MYW) sperm whale myoglobin (20 mg), (RIB) ribonuclease (40 mg, 5 × crystallized) and (CYT) cytochrome *c* (20 mg). Major components of the proteins indicated in the patterns.

direct densitometry of the focused proteins in the gel layer. Increasing amounts, corresponding to 25, 50, 100 and 200 mg protein were applied on the plate on Sephadex G-200 layers of 1 and 2 mm thickness. These conditions were chosen to check whether it is possible to proceed from analytical thin-layer isoelectric focusing to preparative separations just by increasing the thickness of the gel layer. The densitograms convincingly demonstrate that the excellent resolution obtained in analytical separations can be also achieved in preparative focusing, even at the highest protein load in the experiments (Fig. 3). The patterns for a number of the pH marker proteins used in thin-layer isoelectric focusing [16] and the protein mixture in these experiments were almost identical. For some of the proteins e.g. ovalbumin, β-lactoglobulin, and horse myoglobin the patterns resemble closely those obtained by in situ scanning in analytical focusing systems with different anticonvective stabilization [18].

The β-lactoglobulin was included into the mixture to study the effect of isoelectric precipitation in preparative separations. Heavy bands of precipitated β-lactoglobulin were observed in the gel layer during and after focusing, but no adverse influence of the precipitated protein has been noted. The precipitated β-lactoglobulin could be eluted from the gel with a buffer of neutral pH, thus demonstrating the feasibility of isolation of isoelectrically precipitated proteins. When working with granulated gels isoelectric precipitation is not a drawback; it could even facilitate the isolation of some proteins which after precipitation can easily be localized in the gel.

Load capacity

In this work load, capacity is defined as the amount of protein (mg) per ml gel suspension used for preparing the gel layers. The load capacity of a system is obtained by dividing the total protein load by the volume in which the pH gradient is established. Defining load capacity in this way facilitates comparison with other focusing systems which employ a different geometry and/or other forms of anticonvective stabilization. The appearance of a pattern of regular zones was used as the criterion in determining the highest permissible protein load capacity. Overloading resulted first in the formation of irregular zones and a further increase in the amount of protein applied led to marked aberrations in the pattern of zones and the formation of droplets.

Single proteins as well as artificial and natural mixtures of proteins were employed to determine load capacity. By thin-layer isoelectric focusing it has been found that a limit of load capacity appears to exist between 5–10 mg total protein per ml gel suspension. The validity of this finding has been confirmed in small scale preparative experiments on 20 $\times$ 10 cm plates coated with 50 ml gel suspension.

When load capacity for a protein mixture and single proteins are compared for the pH 3–10 and the narrow range ampholytes (Table I) it becomes obvious that in addition to better resolution, the advantage of using narrow range ampholytes is the decidedly higher load capacity for single proteins which increases by a factor of up to 10. Load capacity in narrow pH range ampholytes has been determined for different proteins with major components focused at different pH values. There were distinct differences for the load capacity, e.g. for horse and sperm whale myoglobin, the major components of which were focused at pH 7.35 and 8.15 respectively. These differences probably reflect some non-uniformity of the pH gradient which does not equally well stabilize the focused proteins in all its parts.

TABLE I

LOAD CAPACITY OF ISOELECTRIC FOCUSING IN LAYERS OF GRANULATED GELS (SEPHADEX G-75 AND G-200)

	Protein in gel suspension used for preparing the layer (mg/ml)	
	pH 3–10	Narrow pH ranges
Protein mixture	5– 10	5–10
Single proteins	0.25– 1	2– 4

Table II summarizes load capacity for two frequently encountered fractionation conditions, namely isolation of a major component in presence of minor components and separation of minor components from an excess of a major component. Small scale preparative isoelectric focusing of partially purified egg white proteins has shown that considerably higher load capacities are tolerated when minor components, e.g. conalbumin or lysozyme, are to be separated from an excess of the major component, ovalbumin. Heavy overloading which, for the ovalbumin resulted in distorted zones in the acidic range of the pH gradient, did not disturb focusing of the minor components in the near neutral and alkaline range.

Fractionation of carrier ampholytes

Separation of proteins with very close p*I* values can be expected to be best in a shallow pH gradient. Commercially available ampholytes of two pH units may prove unsuitable for some difficult fractionation tasks, and preparation of carrier ampholytes with narrower pH ranges than those commercially available has been therefore suggested [10, 19]. Layers of granulated gels are particularly suitable for the isolation of narrow range ampholytes. A 20 × 20 cm plate was coated with 100 ml of a gel suspension of Sephadex G-75 containing 10% (w/v) of pH 4–6 ampholytes. After focusing at 300 V for 16 h followed by 2 h at 800 V the pH gradient was

TABLE II

DIFFERENT FRACTIONATION CONDITIONS IN PREPARATIVE ISOELECTRIC FOCUSING

A. Isolation of a major component (ovalbumin) in presence of minor components (conalbumin and lysozyme). B. Separation of minor components (conalbumin and lysozyme) from an excess of a major component (ovalbumin).

	Maximum load capacity (mg protein per ml gel suspension used for preparing the layer)		
Egg white proteins,* partially purified	Ovalbumin (77%)	Conalbumin (20%)	Lysozyme (3%)
Focusing: pH 4–6 and pH 3–10 carrier ampholytes 2/1, by vol.	2–3	10	>10

* Percentage content based on electrophoresis.

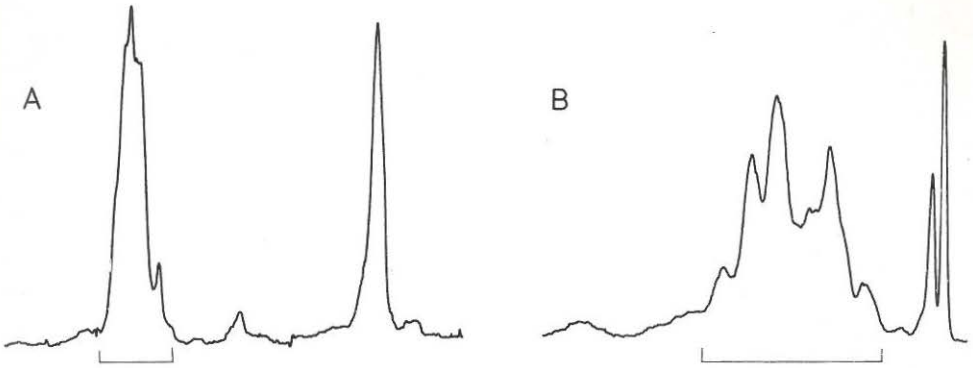

Fig. 4. Thin-layer isoelectric focusing of ovalbumin in pH 4–6 (A) and pH 4.5–4.8 (B) carrier ampholytes. For isolation of the 0.3 pH ampholytes see text. Plates 20 × 10 cm, coated with Sephadex G-75, 0.6 mm layer. Staining with Coomassie Blue G-250. ⌊———⌋ Spread part of the pattern.

determined in the gel layer. From different parts of a single plate, the gel within the range of pH 4–5, 4.3–4.8 and 4.5–4.8 has been removed and diluted at a 1/9 ratio with a Sephadex G-75 suspension containing 0.1 % of pH 6–8 ampholytes. Thin-layer isoelectric focusing demonstrates the dramatic improvement in resolution of ovalbumin in the isolated pH 4.5–4.8 range as compared with the commercial pH 4–6 range (Fig. 4). Ovalbumin consists of several components with very close p*I* values [18, 20] appearing after focusing in pH 4–6 ampholytes as distinct zones in the print but not as separate peaks in the densitogram. The isolated narrow range ampholytes of 0.5 and 1 pH unit gave patterns with a spreading intermediate between that of the unfractionated pH 4–6 ampholytes and the 0.3 pH range ampholytes.

Preparative isoelectric focusing on 40-cm plates

At equal load capacity, use of longer plates could be anticipated to provide in addition to improved resolution a further increase of total load. Preparative separation of 0.5 g sperm whale myoglobin is shown in Fig. 5. Comparison of the pattern with that of Fig. 2 is instructive because the only difference in both experiments lies in the separation distance. As a result of doubled spreading the separation of some minor components has decidedly improved. The greater the distance between the separated fractions the easier their removal from the gel layer and the smaller the risk of contamination from adjacent fractions. Elution profiles of myoglobin after focusing in a density gradient, demonstrate that much of the resolution visible in situ in the column is lost [16, 21]. In granulated gels the myoglobin is not only separated with unprecedented resolution of the minor components even at comparatively high protein loads, but all these components can be isolated from the gel layer without remixing. Most of the components were brown coloured corresponding to metmyoglobin. Although the myoglobin was oxidized with potassium ferricyanide before focusing, a few brightly red coloured fractions corresponding to oxymyoglobin could also be seen in the gel layer and in addition faint fractions of greenish-brown appearance were also visible. Protein staining of the print with Light Green SF, shown at the bottom of Fig. 5, essentially paralled the distribution of the unstained fractions with better differentiation of some of the minor components. On analytical refocusing the

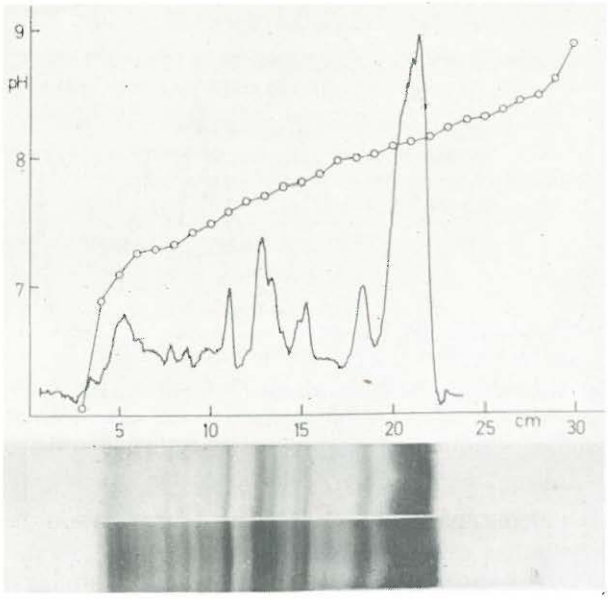

Fig. 5. Preparative isoelectric focusing of 0.5 g sperm whale myoglobin on a 40 cm plate. Sephadex G-200, pH 7–9 carrier ampholytes. Load capacity 2 mg/ml gel suspension. Focusing: 600 V for 16 h followed by 800–1000 V for 8 h. Densitogram of the unstained print, at the bottom-print stained with Light Green SF. ○—○, pH gradient.

myoglobin fractions usually appeared heterogeneous, obviously as the result of a reversible in situ conversion of metmyoglobin to oxymyoglobin, an effect also observed in density gradients [21]. Most other proteins isolated by preparative isoelectric focusing were found to be essentially homogeneous on analytical refocusing, even when amounts greatly exceeding those optimal for staining were applied (see ref. 14, Fig. 10).

Preparative isoelectric focusing of 1.2 g Pronase E, a highly heterogeneous proteolytic enzyme [22–28] obtained from *Streptomyces griseus*, in pH 3–10 ampholytes on a 40 × 20 × 0.2 cm layer is shown in Fig. 7 together with an analytical separation. Pronase E was resolved into more than 25 stainable protein zones which focused between pH 4–9. Total protein recovery based on $A_{280\ nm}$ measurements was over 80%. To determine protein recovery, a 35 × 2 cm gel strip was removed lengthways from the gel layer and the proteins were eluted from the gel with water. This approach in which all proteins were eluted simultaneously from a continuous strip of gel permits a more reliable estimate of recovery than the usual procedure in which protein recovery is calculated by summation of the protein content of individual isolated fractions. The latter method involves loss of the protein between the zones and the inevitable loss of some protein resulting from manipulation of many small fractions. The recovery of proteolytic activity, determined in a similar way, ranged from 40 to 80% depending on the experimental conditions. Experiments at load capacities ranging from 0.5–10 mg Pronase per ml gel suspension have shown that the recovery of proteolytic activity increased with increasing load capacity from 14 to 80%. The highest recovery was repeatedly found at load capacities of 5–10 mg

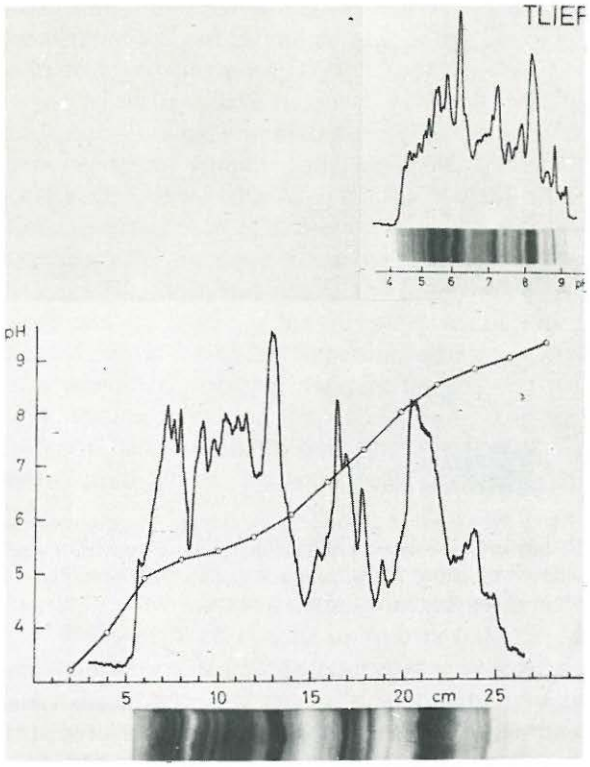

Fig. 6. Preparative isoelectric focusing of 1.2 g Pronase E in a 40 × 20 × 0.2 cm layer of Sephadex G-75 and pH 3–10 carrier ampholytes. Load capacity: 6 mg protein/ml gel suspension. Focusing: 400 V for 16 h followed by 800 V for 6 h. Densitogram of the print stained with Light Green SF (shown at the bottom of the Fig.). ○—○, pH gradient. Thin-layer isoelectric focusing (TLIEF) of Pronase E in Sephadex G-75 and pH 3–10 carrier ampholytes. Load capacity: 5 mg protein/ml gel suspension. Protein staining with Coomassie Brilliant Blue G-250.

Pronase per ml. Since the caseinolytic activity of Pronase is inhibited by EDTA [22], inactivation of Pronase following isoelectric focusing could be due to the chelating properties of the carrier ampholytes [29]. The dependence of enzyme recovery on load capacity and therefore also on the ratio of enzyme to the carrier ampholytes is compatible with this explanation. However, addition of calcium [23] after focusing did not improve the recovery of enzyme activity.

Large scale preparative isoelectric focusing

For preparative focusing of gram quantities, a trough 40 × 20 cm with a gel layer up to 1 cm and a total volume up to 800 ml has been used. The pattern after focusing of 10 g Pronase E (Fig. 8) demonstrates the excellent resolution obtainable at a load capacity of 12 mg/ml. There were no indications of overloading for any of the proteins thus proving the effectiveness of anticonvective stabilization by granulated gels also in 1 cm layers. Some difficulty was experienced in obtaining adequate destaining of the print shown in the lower part of Fig. 8. As in the preceding experiments at high load capacity numerous clearly separated transparent zones could be

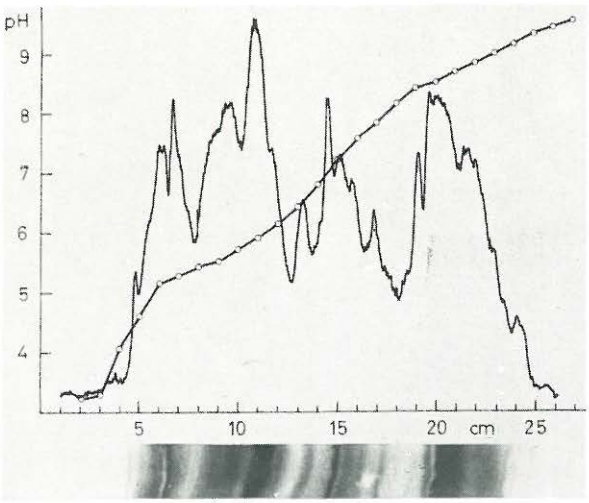

Fig. 7. Preparative isoelectric focusing of 10 g Pronase E in a 40 × 20 cm trough. Thickness of the gel layer: 1 cm. Sephadex G-75, pH 3–10 carrier ampholytes. Load capacity: 12 mg protein/ml gel suspension. Focusing: 300 V for 20 h followed by 800 V for 6 h. Densitogram of the print stained with Light Green SF (shown at the bottom of the fig.). ○—○, pH-gradient.

seen directly in the gel layer. Also at this very high total protein load and high load capacity the pattern closely resembled that of analytical separations. On analytical refocusing some of the Pronase fractions were homogeneous, while others proved to be heterogeneous, with additional components appearing in a part of the pH gradient rather distant from the original position of the refocused fraction. Autolytic degradation of the proteolytically active fractions could offer an explanation for this finding.

DISCUSSION

Although the potential of preparative isoelectric focusing has been early recognized [12] surprisingly few applications have been described until now. The success of preparative isoelectric focusing will depend on such factors as anticonvective stabilization, geometry of the focusing system, heat dissipation, load capacity resolving power and recovery. The two main methods of anticonvective stabilization used at present are density gradients and gels. Use of a density gradient for preparative separations in vertical columns has several limitations: low load capacity [4], sensitivity to local temperature fluctuations [1], difficulties in ensuring efficient cooling which imposes finite limits on the column design, isoelectric precipitation [4, 12], and loss of resolution due to remixing of proteins on elution from the column [1, 16, 21]. Some of these drawbacks can be overcome by continuous flow density gradient systems [13] or by short vertical columns [30].

A high load capacity, in terms of the amount of protein per cm^2 of a single zone, has been demonstrated for cylindrical polyacrylamide gels [7], but due to the unfavourable geometry these gels cannot be used for preparative separations of higher quantities of protein. The geometry of a focusing system thus determines the scale

of operation and the limits of scale up. Heat-dissipation depends strongly on the geometry of the system and decides on the use of high voltage gradients which in turn will effect resolving power.

When used for anticonvective stabilization of the pH gradient in preparative isoelectric focusing, granulated gels offer a number of advantages. Chemically different granulated gels, commercially available either as dextran, e.g. Sephadex, or polyacrylamide gels, e.g. Bio-Gel, can be used without the need for polymerization thus eliminating the risks of artifacts caused by polymerization catalysts [16]. As in thin-layer isoelectric focusing it has been found that the Sephadex gels are handled easier than the Bio-Gels, but use of the latter could be justified in those applications in which contamination of the focused proteins with traces of carbohydrates has to be avoided. Sephadex G-200 Superfine appears to be the best supporting gel for preparative isoelectric focusing yielding the greatest spreading of proteins with extreme p*I* values and tolerating the highest load capacity. With Sephadex G-75 it is usually simpler to get a print [16] but with papers of sufficiently high surface weight a satisfactory print can be obtained also from gel layers of the higher G-numbers of Sephadex.

The geometry of a layer is favourable for heat dissipation, thus permitting use of high voltage gradients, a factor important for resolution. In most preparative experiments focusing was started with voltage gradients of 10–15 V/cm and terminated at 20–40 V/cm. Watt charges up to 0.05–0.06 W/cm^2 were well tolerated by layers up to 1 cm thickness and heat dissipation was efficient enough to avoid local overheating. A factor of great practical value is the free accessibility of the gel layer for sample application and during focusing for pH measurements. Small samples can be removed from the gel layer during focusing, e.g. for enzyme determinations.

For single proteins load capacities of the order of 0.25–1 mg per ml gel suspension have been estimated for the pH 3–10 ampholytes and 2–4 mg per ml for the narrow range ampholytes of two pH units (Table I). Much higher figures for load capacity have been found for some special separation conditions, e.g. the isolation of a minor component in the presence of a large excess of other proteins (Table II). Provided that the p*I* values of the major components to be separated are sufficiently spaced in the pH gradient and the quantities of the individual major components do not differ too strongly, total loads capacities in the order of 5–10 mg/ml have been estimated and experimentally verified for the pH 3–10 and the narrow pH range ampholytes. An approach to the separation of proteins with very close p*I* values has been outlined in an experiment in which ovalbumin has been focused in a 0.3 pH cut of carrier ampholytes obtained by focusing of ampholytes at a concentration of 10% (Fig. 4). High load capacity, however at the expense of resolution, has also been described for some focusing experiments in a density gradient [3, 5], thus qualifying the method only for a rough fractionation. A very convincing illustration of the efficiency or preparative isoelectric focusing at high load capacity in layers of granulated gels is given in Fig. 3. In this experiment 200 mg of protein have been separated on a quartz plate in a gel layer with a volume of only 50 ml which is approximately half the volume of the most frequently employed column for focusing in a density gradient [4].

Comparison with different focusing systems could be achieved by expressing load capacity in terms of mg protein per ml of "focusing volume". Due to lack of

sufficient data, load capacity of layers of granulated gels cannot be compared directly with most other approaches suggested for preparative isoelectric focusing. Continuous flow systems have been suggested for preparative focusing of high quantities of proteins [13]. The Sephadex stabilized system is claimed to permit much higher loads than the density gradient method, for which a load capacity of 2.5 mg total protein per ml has been calculated for focusing of a mixture of three proteins. High load capacity in preparative focusing should prove important not only with respect to economical considerations of this separation process. A high ratio of protein to carrier ampholytes improves the recovery generally, and in particular the recovery of enzyme activity in those instances in which an inactivation occurs due to the chelating activity of the carrier ampholytes [29]. As an added operational advantage, high load capacity facilitates isolation of the focused proteins which even in the case of uncoloured proteins can be seen in the gel layer as transparent zones.

Recovery of proteins after focusing is high and elution is very simple. The focused proteins are usually obtained in a small volume and relatively high concentrations, thus permitting analytical refocusing of most isolated fractions without further manipulation. Based on optical measurements, with appropriate corrections for the absorbance of the carrier ampholytes, a recovery of 80–90% has been found for single proteins and protein mixtures. The absence of sucrose, or other solutes used in density gradients, in the eluates from granulated gels is a definite advantage on later removal of the carrier ampholytes.

The total amount of protein separated by preparative isoelectric focusing in Sephadex layers of granulated gels ranged from 0.1–10 g. These figures reflect the versatility of the method. Scaling up of preparative isoelectric focusing could be achieved just by variation of the length, width, and thickness of the gel layer. The flexibility of the system permits the dimension of the gel layer to be optimally adapted to the amount of protein to be separated. An upper total load of 10 g protein has been described in this paper but it seems feasible to scale up the method for higher quantities of protein.

Preparative isoelectric focusing in layers of granulated gels opens new perspectives for the isolation and purification of proteins. The high resolving power of analytical isoelectric focusing in layers of granulated gels is attained also in preparative applications under conditions of great experimental simplicity. The significance of these results is evident when considering the difficulties experienced in passing from analytical polyacrylamide-gel electrophoresis to a preparative scale. Although gram preparative apparatus have been already described [31, 32], preparative polyacrylamide-gel electrophoresis still appears to be in a stage of development. The many different devices for separation in a miligram scale reflect the difficulties in optimizing the method for preparative purposes [33]. For some fractionations, preparative isoelectric focusing in layers of granulated gels could prove to be the method of choice when employed as a single step. Most efficient purifiction can be anticipated to be achieved when methods based on different molecular parameters of the separated substances are employed, an aspect which will be treated in detail elsewhere [34].

ACKNOWLEDGEMENTS

I am indebted to Professor J. F. Diehl for generous support and to Dr H.

Delincée for helpful discussions. The skilful technical assistance of Mrs L. Zimmermann and A. Sieron is gratefully acknowledged.

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