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FRACTIONATION OF CARRIER AMPHOLYTES FOR ISOELECTRIC FOCUSING

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SUMMARY

A simple method for fractionating synthetic carrier ampholytes is reported, based on the principle of continuous-flow isoelectric focusing in gel-stabilized layers. An 8% ampholyte solution, encompassing the pH range 3–9.5, is separated into 12 fractions in a chamber filled with Sephadex G-100 by a continuous-flow technique. We are thus able to obtain ampholytes of narrow pH range, encompassing approximately 2 pH units, whose resolving power is comparable with that obtained with commercial Ampholine covering similar pH ranges.

INTRODUCTION

In the preceding paper[1], we described the synthesis of carrier ampholytes suitable for isoelectric focusing. Acrylic acid is coupled to either hexamethylenetetramine (HMTA) or triethylenetetramine (TETA), or tetraethylenepentamine (TEPA) or pentaethylenehexamine (PEHA), using a nitrogen:carboxyl ratio of 2:1. The reaction has a yield greater than 98%. HMTA, TETA, TEPA and PEHA ampholytes are pooled to form a mixture of a great number of amphoteric compounds with closely spaced pKs and pIs in the pH range 3–9.5. This mixture produces smooth and stable pH gradients, and does not show any conductivity gap along the pH gradient formed. Detection of focused ampholytes along a gel slab, by a refractometric method, reveals sharp ampholyte gaussians closely spaced all along the separation support medium[1].

In this paper, we report the separation of these ampholytes, encompassing the pH range 3–9.5, into narrower pH ranges, covering 1–2 pH units. This fractionation technique is an electrophoretic method based on the continuous-flow system described by Fawcett[2].

MATERIALS AND METHODS

Sephadex G-100 was obtained from Pharmacia, Uppsala, Sweden. Samples of bovine pepsin, pig pepsin, chick pepsin and rennins from *Mucor mihei* and *Mucor pusillus* were kindly supplied by La Biotecnica, Trento, Italy.

The synthesis of carrier ampholytes was described in the preceding paper[1]. Isoelectric focusing in polyacrylamide gel slabs has been reported previously[3]. Continuous-flow isoelectric focusing was carried out essentially as described by Fawcett[2]. More details are given under Results.

RESULTS

Fig. 1 depicts the arrangement for continuous-flow isoelectric focusing of ampholytes. The separation chamber (D), 14 × 0.6 × 22.5 cm, is made of Plexiglass. The chamber has cooling jackets built in on both sides and is cooled by circulating water at 1° from a thermostat (A). To the bottom of the chamber are glued twelve outlet channels (3 mm I.D.) evenly spaced at 1-cm intervals. Against the bottom, from the inside of the chamber, is pressed a strip of porous polyethylene (average pore size 50 μm) to prevent drainage of the resin. On the two sides of the chamber are built the electrode compartments, 23 × 0.7 × 2.3 cm, which are filled with 1 N sodium hydroxide solution at the cathode and 1 M orthophosphoric acid at the anode. The electrical connections are made of platinum wire wound around two lucite strips, in widely spaced turns, going to the bottom of the electrode compartments. The anodic and cathodic vessels are sealed off from the separation chamber, except for the last
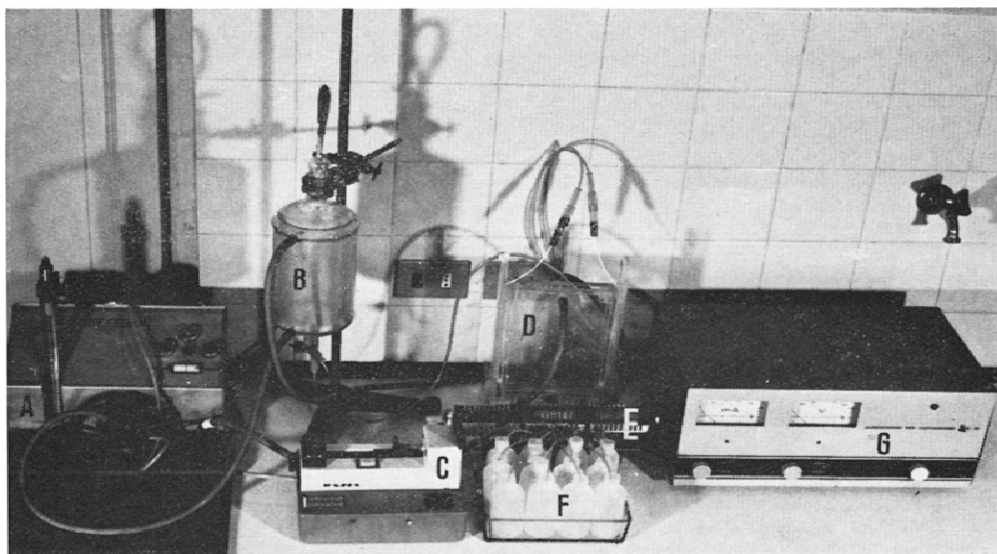

Fig. 1. Equipment for continuous-flow isoelectric focusing. A, Thermostat; B, Mariotte flask (from an LKB Uniphor column); C, stepping motor from a Razel syringe pump, Model A99; D, chamber for continuous-flow isoelectric focusing; E, modules of a Delta/6 pump (Watson-Marlow); F, collection bottles; G, power supply.

3 cm at the bottom, where the plastic wall is replaced with two rectangular pieces of porous polyethylene (2 × 3 cm), in order to allow electrical conductivity. The separation chamber is filled with 140 ml of a Sephadex G-100 slurry, swollen in a solution of 8 % ampholyte mixture to be fractioned, and de-gassed. A 3-cm depth of free ampholyte solution is allowed above the gel surface.

Immediately after pouring the gel slurry, the electrode chambers are filled with anolyte and catholyte to the same liquid level as in the central chamber. The fractionation chamber is connected, via a plastic tube, to a Mariotte flask containing the same 8 % ampholyte mixture to be fractionated. The Mariotte flask (taken from an LKB Uniphor column) is filled with 1.5 l of 8 % ampholyte solution, and is fitted with a cooling jacket through which water at 1° from a thermostat is circulated. As the liquids in the reservoir and in the fractionation chamber are hydrostatically equilibrated, constant automatic sample feeding is provided, without any need for pumps. The twelve outlets of the chamber are connected with silicone rubber tubing to Delta/6 pump modules (from Watson-Marlow, Falmouth, Cornwall, Great Britain) that are driven by the stepping motor of a Razel Model A99 pump (Razel Scientific Instruments, Stamford, Conn., U.S.A.). The motor was removed from the Razel pump and connected mechanically to the axle of the Delta pump modules. This arrangement provided a stable, digitally pre-set flow-rate from 1 to 90 ml/h (total flow from twelve channels). The tightening of the tubing around the Delta pump is adjusted so that they deliver equal volumes. No recirculation of anolyte and catholyte, as suggested by Fawcett[2], is provided and therefore in the separation chamber the field strength rapidly decreases from the porous polyethylene membranes toward the top of the chamber.

At the start, a voltage of 200 V, with a corresponding current of 70 mA, is applied and the Delta pump is not activated. After 3 h, the voltage has increased to 300 V and the current has dropped to 50 mA. The zones of "chromophoric" ampholytes are now seen as vertical, focused bands. At this stage the Delta pump is started at a speed of 20 ml/h (total flow from twelve channels) and the fractions are collected in twelve plastic 100-ml bottles. The system then runs at a steady state, and can be left unattended.

Fig. 2 shows details of the central system, consisting of the electric motor (A), Delta units (C), separation chamber (B) and collection bottles (D), kept in a glass basin. This type of Delta pump can be run with many more channels, as blocks of ten channels can be linked together in a linear fashion to the electric motor. In Fig. 2 are shown three blocks of ten channels each (C), interposed between the fractionation chamber (B) and the collection bottles (D).

Fig. 3 shows the pH profiles in the twelve collection bottles when the system was run for 10 days without interruptions. Every 24 h the bottles were harvested and the pH values measured at room temperature. It can be seen that the pH is constant in each fraction, except for fraction 7, which shows an initial pH drift during the first 70 h. That the system was at equilibrium was also ensured by the constancy of the voltage and the current across the separation chamber. Also, the positions of the yellow lines of "chromophoric" ampholytes, marked on the wall of the chamber, did not shift during the experimental period (10 days). The separation shown in Fig. 3 was achieved from an 8 % ampholyte solution, obtained by mixing HMTA, TETA, TEPA and PEHA ampholytes in a ratio of 1:2:2:3. All of these ampholytes were synthesized
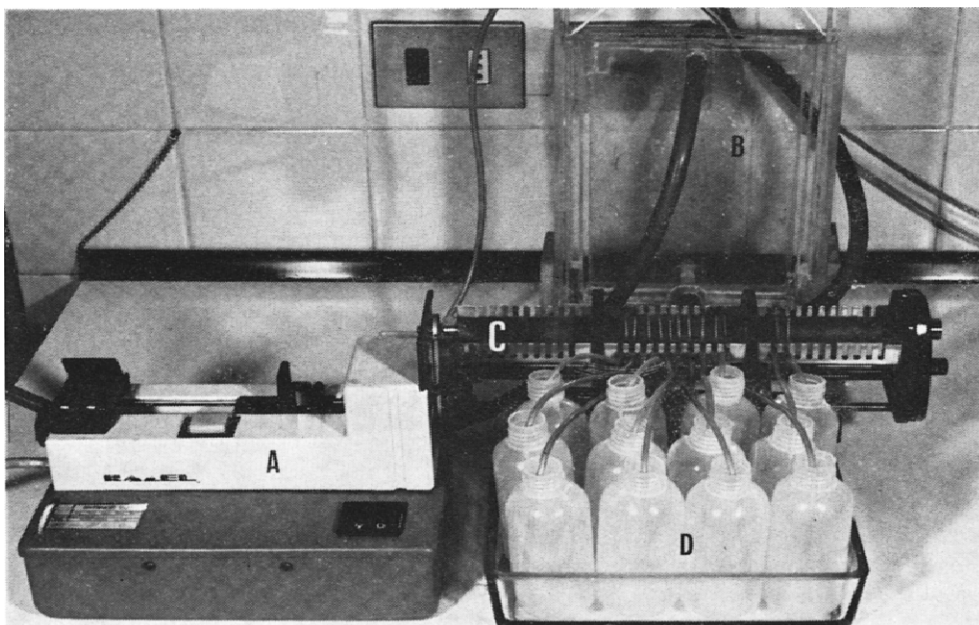
Fig. 2. Details of the central unit for continuous-flow isoelectric focusing. A, Stepping motor from a Razel syringe pump, with digital control unit for flow-rates; B, fractionation chamber for continuous-flow isoelectric focusing; C, blocks of three Delta units linked together, with a capacity for 30 channels (only 12 connected); D, collection bottles for the 12 fractions.
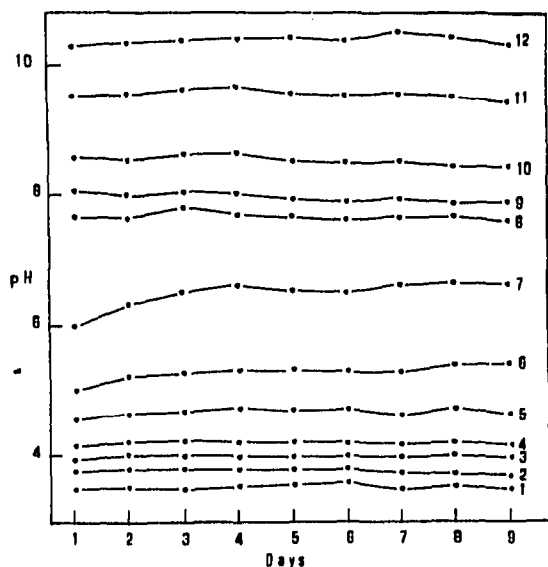

Fig. 3. Continuous-flow fractionation of a mixture of HMTA, TETA, TEPA and PEHA ampholytes in the ratio 1:2:2:3 for 9 days. The PEHA ampholytes were synthesized with a nitrogen:carboxyl ratio of 1.5:1 and with a nitrogen:carboxyl ratio of 1:1. The various curves give the pH in each fraction at 24-h intervals. The numbers on the right of each curve indicate the fraction number (from anode to cathode).

with a nitrogen:carboxyl ratio of 2:1, except for PEHA ampholytes, which were obtained from a batch with a nitrogen:carboxyl ratio of 1.5:1 and from a batch with nitrogen:carboxyl ratio of 1:1. This explains why so many fractions (bottles 1–6) contain acidic species in the pH range 3–5.5 (see also Fig. 3 in ref. 1).

We then tested the various fractions by polyacrylamide gel isoelectric focusing, in order to obtain the actual pH ranges encompassed by the separated ampholytes. Fractions 1 and 12 were discarded, as they were shown to be contaminated with orthophosphoric acid and sodium hydroxide, respectively. Fractions 2–6 were pooled in a single ampholyte batch, as they had p*I* values too close to one another and, separately, they would probably have formed pH gradients too shallow to be of practical use. The other fractions were used as such.

Fig. 4 gives the results of these experiments. The black triangles are the average pHs as measured in each fraction eluted by the continuous-flow technique. The vertical lines represent the actual pH gradient generated by the various fractions upon focusing in polyacrylamide gel. In this way, we were able to collect six different fractions, each covering a range of approximately 2 pH units, encompassing the pH range 3–9.5.
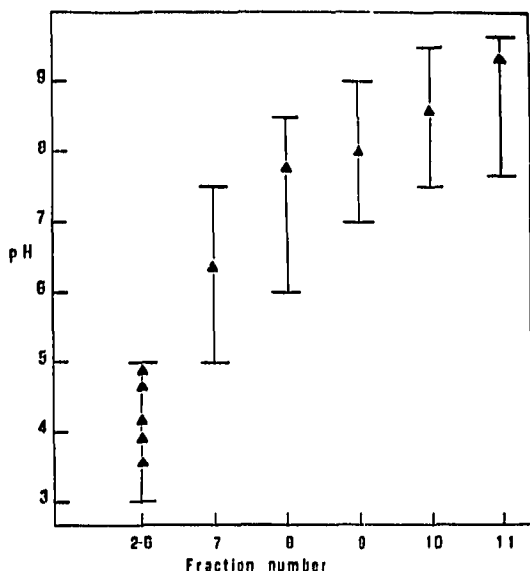

Fig. 4. Actual pH range of each fraction separated by continuous-flow isoelectric focusing. The black triangles indicate the average pH in each collection bottle, and the vertical lines the pH range generated by the various fractions upon isoelectric focusing in polyacrylamide gels. Fractions 1 and 12 were discarded, as they were contaminated by anolyte and catholyte, respectively. Fractions 2–6 were pooled.

Fig. 5 shows the separation of some commercial rennets in the acidic pH range, using the pooled fraction 2–6 from Fig. 4. In this particular case, as we wanted also to detect the four components in bovine pepsin, which are isoelectric around pH 2.6, one third of the ampholytes in the mixture was represented by LKB Ampholine "pH 2.5–4". Without it, except for the loss of bovine pepsin, the separation would
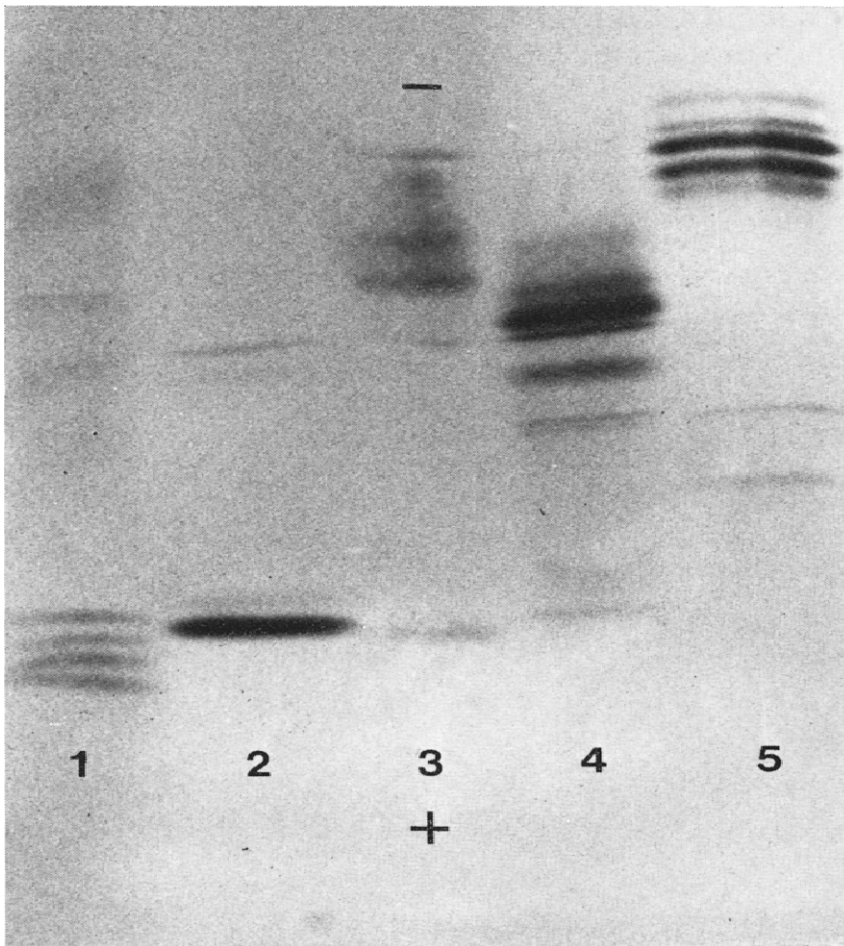
Fig. 5. Isoelectric fractionation of rennins in a polyacrylamide gel slab. As ampholytes we used a mixture of two thirds of the synthetic product (fractions 2–6 of Fig. 4) and one third of LKB Ampholine "pH 2.5–4". Samples: 1, bovine pepsin; 2, pig pepsin; 3, chick pepsin; 4, rennins from *Mucor pusillus*; 5, rennins from *Mucor mihei*.

have been identical. In fact, even pig pepsin would have been detected as a sharp band at the edge of the filter-paper strip of the anode. In any event, the number of components found and their respective p*I*s were the same as those obtained by performing the separation in a mixture of commercial Ampholine "pH 2.5–4" and "pH 4–6". The separation of rennins will be described elsewhere[4]. We have produced protein patterns with other ampholytes of narrow pH range fractionated by us (for instance, the separation of haemoglobins in fraction 8 and of human ferritins in fraction 7) and found an excellent correlation with the patterns obtained with commercial Ampholine encompassing similar pH ranges.

DISCUSSION

The present ampholyte fractionation system appears to offer several advan-

tages over multi-compartment electrolyzers, as described by Vesterberg[5] and Rilbe[6]. The latter apparatus seems to be difficult to build in an ordinary laboratory workshop. It is usually constructed with 20 cells and each cell has to have an independent stirring system. The fitting of the single cells with outer cooling jackets or inner cooling systems also appears to be difficult, as the entire apparatus should be built in such a way as to allow dismantling into single units. The membranes between pairs of cells have to be changed often and, during electrolysis, they can also give rise to undesirable polarization effects.

Another drawback of multi-compartment electrolyzers is the phenomenon of unbalanced osmotic pressure. If, during isoelectric focusing, one cell becomes particularly enriched in ampholytes, compared with neighbouring cells, this cell will show an increase in osmotic pressure. In order to counteract this effect, water will be drawn in until the osmotic pressure is balanced again. This phenomenon will adversely affect the pattern of focused ampholytes.

A further disadvantage of this system is that, at the end of the run, the electrolyzer has to be dismantled, cleaned, the membranes changed, and re-assembled for another run.

All of these problems are eliminated in our continuous-flow system. Once in operation, it can be run for several weeks and left unattended without risk, provided that adequate sample input is ensured. Assembly and dismantling of the equipment shown in Fig. 1 is easy. Gel stabilization of focused ampholytes during isoelectric focusing appears to be superior to membrane stabilization against convection and diffusion. Another advantage of our continuous-flow system is that the ampholytes, during the fractionation process, are kept in contact with the anolyte and catholyte only for a short time (*ca.* 1 h and 15 min respectively, at the flow-rate used). This prevents anodic oxidation and chemical modification of ampholytes. In multi-compartment electrolyzers, where the ampholytes are kept in the apparatus for at least 24 h, Vesterberg[5] reported the formation of yellow compounds, spreading from the anode. In order to prevent anodic oxidation, special precautions had to be taken[5].

We found it adequate, for our fractionation purposes, to have a twelve-channel outlet. However, if a finer resolution of ampholytes is needed, with collection of more fractions, our apparatus can easily be modified with interchangeable bottom outlets, containing as many channels as needed. Fawcett[2] (who built the continuous-flow chamber we have been using) reported fractionation of proteins in chambers containing as many as 54 channels.

We hope that the ease with which suitable carrier ampholytes can be synthesized and fractionated in narrow pH ranges, in a biochemical laboratory, will stimulate more research and applications in the field of large-scale preparative isoelectric focusing.

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