ISOELECTRIC FOCUSING OF PROTEINS ON BACTOPEPTONE

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Received November 18th, 1970

The applicability of commercial bactopeptone Spofa as the ampholytic carrier for isoelectric focusing of proteins was studied. The focusing ability and resolving power of bactopeptone was examined both in a liquid column with a pH gradient stabilized with a sucrose gradient, and in microcolumns stabilized with polyacrylamide gel. In both techniques the bactopeptone solutions yield a continuous pH gradient in the effective range from 3 to 9 without nonconductive zones. Practical examples of bactopeptone application to focusing of haemoglobins and haemolyzates of human erythrocytes are shown, indicating a high resolution power of the bactopeptone pH gradient.

Isoelectric focusing in natural pH gradients was applied and theoretically founded as a highly effective method for protein separation by Svensson and Vesterberg[1-5]. The practical application of the method met with difficulties for lack of suitable ampholytic carriers until a series of homologues and isomers of polyaminopolycarboxylic acids was synthesized, by coupling acrylic acid with various polyethylene-polyamines[6] which satisfied the theoretical requirements formulated previously by Svensson[2]. The mixtures are marketed under the name Ampholines (Uppsala, Sweden) for different pH ranges and are the only existing carrier for isoelectric focusing presently used. At first, Svensson used a mixture of low-molecular ampholytes of a different chemical nature, among others also hydrolyzates of proteins. The results were not satisfactory: the peptide carriers formed coloured zones during focusing, the higher oligopeptides present were only slowly separated from proteins by dialysis and, furthermore, the isoelectric carriers at about pH 6 were not properly represented in the mixture so that, due to low conductivity, a thermal convection occurred in this region.

In spite of these apparent drawbacks we set out to check the limits of applicability of readily available and inexpensive bactopeptones as carrier ampholytes. The results showed that a simple process turns the local bactopeptone (Spofa, Prague) into an ampholytic carrier with a high resolving power, well applicable to isoelectric focusing of proteins.

EXPERIMENTAL

Isoelectric focusing was studied using a liquid column stabilized with a density gradient (24 × × 245 mm, 110 ml), and microcolumns (5 × 65 mm) stabilized with a polyacrylamide gel. In the first case we used an apparatus resembling that described by Svensson[3] usually with 1% bactopeptone. 5·3 g bactopeptone Spofa was dissolved in 26 ml distilled water, 70 ml 96% ethanol

was added, the mixture left to stand for 2 days and filtered. Ethanol was then removed by concentrating the mixture to 1/5 original volume, the amount made to 100 ml and the solution filtered again. The stock solution was preserved with a drop of chloroform. The pH gradient was stabilized with a sucrose gradient prepared as described before[4]. The central column of the apparatus containing the anode was filled with distilled water made acid with a few drops of acetic acid; the cathode terminal component was ethylenediamine added in the amount of a few drops to the last, least dense, layer of the column. The focusing instrument was attached to direct current from a Tesla BS-275 source which was manually controlled to keep the maximum input below 2 W. The initial values of 200 V and 10 mA were gradually brought up to 700 V at about 2 mA. One hour before and in the course of the focusing the column was cooled by running tap water (13°C). After focusing, the column content was let out at a rate of 2 ml/min, taking 5 ml fractions.

Microfocusing in a polyacrylamide gel was carried out by a slightly modified method described for microfocusing with Ampholines[7] using an apparatus for disc electrophoresis[8]. The polymerization mixture (3·0 g Cyanogum 41, made by American Cyanamid, which is an acrylamide containing 5% N,N'-methylene-bis-acrylamide, 6 ml 5% bactopeptone and 1 ml 0·01% riboflavine, made to 30 ml with distilled water) was placed in glass tubes (80 X 5) up to a 65 mm mark and, after overlayering with water, left to polymerize in diffuse daylight. In most experiments, the polymerization mixture contained 1·5 g sucrose which improves the quality of the gel. After polymerization, the liquid layer was removed by suction, the tubes filled to the rim with the catholyte (0·5% ethylenediamine; the anolyte was 0·3% sulfuric acid) and underlayered with a sample containing 10% sucrose. After placing into the apparatus, the initial current was adjusted below 2 mA per tube (about 150 V with 10 tubes). The voltage was then gradually raised to 350 V and then left to drop to the limiting value of about 0·3 mA per tube). After this value had been reached, the focusing was interrupted and the column stained with bromophenol blue[9] (0·2 g bromophenol blue, 50 ml 96% ethanol and 5 ml glacial acetic acid with water added to 100 ml). The bleaching solution contained 35% aqueous ethanol and 5% acetic acid. The column with bactopeptone without protein yields an optically clear background.

TABLE I

Values of pH, Conductivity and Nitrogen Content in Effluent Fractions

Five ml fractions were collected from a 110 ml column, after 22·5 h of focusing. 1% bactopeptone.

Fraction	pH	$\varkappa . 10^{-4}$	mg N	Fraction	pH	$\varkappa . 10^{-4}$	mg N
1	3·26	2·75	3·48	11	5·27	0·19	3·95
2	3·07	2·65	3·88	12	5·50	0·17	4·40
3	3·04	1·81	3·70	13	5·78	0·14	4·30
4	3·03	1·72	4·25	14	6·18	0·17	4·63
5	3·05	1·72	3·72	15	7·14	0·30	5·00
6	3·11	1·49	3·60	16	7·60	0·37	3·18
7	3·43	1·05	4·17	17	8·52	0·77	3·23
8	4·12	0·81	6·45	18	9·55	3·28	4·77
9	4·64	0·47	6·60	19	10·10	4·52	6·45
10	5·05	0·27	4·87	20	11·30	9·20	6·72
				21	11·50	26·40	4·83

The pH *was determined* with a Radiometer PHM 4 and a glass electrode. Titration curves were obtained by potentiometric microtitration with 1M-NaOH of 5% solutions of bactopeptones or of 2% Ampholine (pH 3–10). The pH gradient of the liquid column stabilized with sucrose was measured in 5 ml fractions of the effluent. In polyacrylamide gels the pH gradient was measured in such a way that the columns were cut into 2 mm discs, the sections placed in 0·5 ml distilled water in small dishes, sealed with an adhesive tape and left for 24 h at 7°C. The pH was then estimated.

The conductivity of effluent fractions was calculated from the resistance of solutions estimated with a Conductoscope and a conductivity vessel standardized with 0·100M-KCl at 25°C. The nitrogen of the fractions was determined spectrophotometrically (Specol) by Nessler's reagent after ashing with sulfuric acid and hydrogen peroxide.

RESULTS

It is essential for satisfactory function of carrier ampholytes that they possess sufficient buffering power, conductivity and uniform distribution along the pH gradient. From this point of view the bactopeptones were characterized in this work. Fig. 1 shows the titration curves of three commercial bactopeptones which characterize the buffering power (dpH/dNaOH). For the sake of comparison, the buffering power of Ampholine (pH 3–10) is shown in the same scale. For further studies we selected bactopeptone Spofa. In comparison with the Ampholines, it has a somewhat lower buffering power, particularly in the acid region. Table I shows the values of pH,

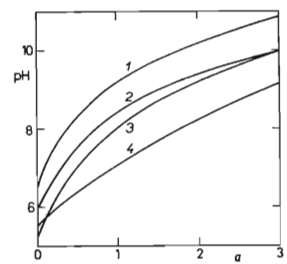

FIG. 1

Titration Curves of Bactopeptones and Ampholine (pH 3–10)

Peptones: *1* Difco, *2* Merck, *3* Spofa, *4* Ampholine. The ampholyte content in the Ampholine was calculated from the concentration given by the producer. *a* mequiv. hydroxide/g carrier.

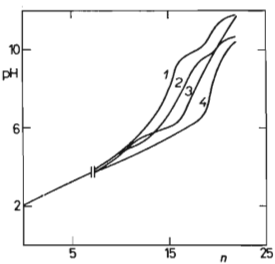

FIG. 2

Changes in the pH Gradient in a Column of 1% Bactopeptone during Focusing

1 20, *2* 24, *3* 43, *4* 60 h focusing at 700 V. *n* Number of fraction.

conductivity, and nitrogen concentration in 5 ml fractions of the effluent from a 110 ml column of bactopeptone after 22·5 h of focusing. The pH gradient was stabilized with a density gradient so that the separation parameters of the ampholytic carriers are similar as during electrofocusing of proteins. The course of the parameters along the column is characterized by a continuous pH gradient, by a uniform distribution of the carrier ampholytes and by the absence of nonconductive zones (higher values of conductivity at both ends of the column are due to the added catholyte and anolyte. In the first experiments, a certain drawback of bactopeptone became apparent. During the focusing, turbidity zones were formed at several places together with droplet sedimentation. This was removed by extracting the bactopeptone with 70% aqueous ethanol. The course of the pH gradient depends on the focusing time (Fig. 2). With longer separation times the alkaline part of the gradient becomes steeper. The lesser efficiency of the bactopeptone in the neutral region of pH may be improved by adding histidine[3] (Fig. 3) to the mixture of carriers. In our experiments with haemoglobin this modification was unnecessary. The behaviour of the bactopeptone carrier was further studied in small columns stabilized with polyacrylamide gel. Fig. 4 compares the gradient formed by 1% bactopeptone and 1% Ampholine (pH 3 – 10). After 90 min of focusing the bactopeptone gradient shows a relatively simple course without steep sections, more or less parallel to that of the Ampholines. An example of the efficiency of bactopeptone during protein focusing in a liquid column stabilized with a sucrose

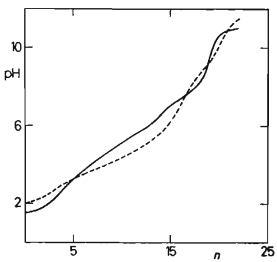

FIG. 3

Change of pH Gradient in the Presence of Histidine

– – – – Focusing of 1% bactopeptone for 24 h at 700 V; ———— the same with 0·11 g histidine per 110 ml carrier solution. *n* Number of fractions.

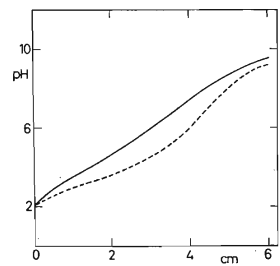

FIG. 4

Comparison of the pH Gradient with Bactopeptone and Ampholine (pH 3—10)

– – – – 1% Bactopeptone, ———— 1% Ampholine.

gradient is shown in Fig. 5*, showing a focused haemolyzate of erythrocytes from umbilical blood, containing Hb A (IP 6·88) and Hb F (IP 6·97). The pH fraction of the effluent containing Hb A had a pH of 7·00 which should be the value of the isoionic point[4]. Separation of Hb A from Hb F indicates that the resolving power of the bactopeptone gradient is 0·1 pH unit. A comparison of the resolving power of bactopeptone with that of Ampholine (pH 3–10) is shown in Fig. 6*, based on a haemolyzate of well-washed human erythrocytes. Both the bactopeptone and Ampholine columns show immediately after focusing a clear heterogeneous zone of haemoglobin, usually composed of two to three components. After staining, further narrow zones appear in the acid region. These zones are not clearly visible in the reproduction in the Ampholine column, apparently due to the longer time required for bleaching – about twice or three times that for bactopeptone. The background of the focused bactopeptone column without protein sample remains optically clear under these conditions. Just as after focusing in Ampholines[10] one can identify the focused proteins on a polyacrylamide column with a bactopeptone carrier by immunochemical reactions, *e.g.* by diffusion from the column placed on an agar plate against specific antisera.

DISCUSSION

It was derived theoretically that the low value of the difference between the isoelectric point and the nearest dissociation constants (pI – pK_1) for pK_1 < pI is an important property of good ampholytic carriers. The value is related to the conductivity of the ampholyte at the isoelectric point as well as to its buffering capacity[2]. For any individual ampholyte the buffering capacity is indicated by the titration curve. Bactopeptone contains perhaps several dozen different peptides. Thus the titration curve of bactopeptone (Fig. 3) itself does not indicate the suitability of an ampholyte since the acid groups are titrated in the sequence of dissociation constants irrespective of their being grouped in the individual peptides. The titration curve yields rough information on the possible range of the pH gradient after focusing separation and, besides, an empirical comparison with other commercial peptones and Ampholines. The analysis of effluent fractions after isoelectric separation of bactopeptone, for nitrogen, conductivity and pH (Table I) indicates a relatively uniform distribution of the peptide components along the direction of the electrical field, a continuity of the pH gradient and the preserved conductivity of the column. Nonconductive zones would bring about local heating during the passage of current, accompanied by thermal convection. These values hold for the given duration of electromigration and for the given current conditions.

The essential property of a "natural" pH gradient is the stable final steady state which ideally is defined in such a way that the ampholytes during focussing assume

* See insert facing p. 326.

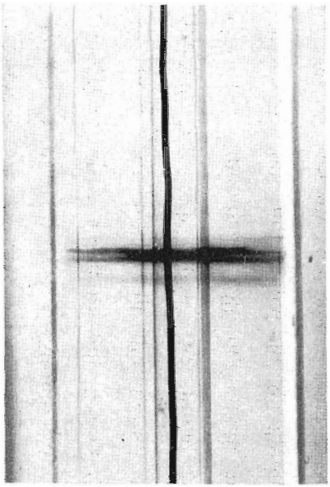

FIG. 5

Isoelectric Focusing of Erythrocyte Haemolyzate from Umbilical Blood: Haemoglobins A and F

1% Bactopeptone, focused for 24 h at 700 V. Five ml washed erythrocytes from citrate blood were haemolyzed with 2 ml water. 0·06 ml placed on the column.

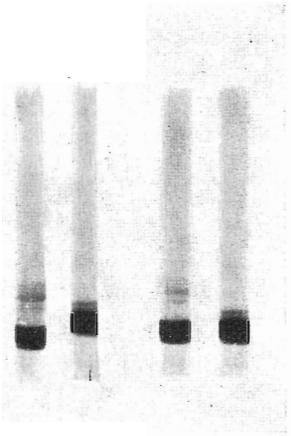

FIG. 6

Focusing of Human Blood Haemolyzate on Bactopeptone and Ampholine (pH 3—10)

The focusing pattern of erythrocyte haemolyzates: Ampholine on the left, bactopeptone on the right.

stable positions along the direction of the electric field in the sequence of their isoelectric points. Their concentration in the isoelectric zones obeys certain distribution curves, the concentration gradients of which are determined by electromigration as a concentrating force and by diffusion as a spreading force. The bell-shaped curves of the concentration distribution overlap and thus passage of electric current is made possible in isoelectric conditions.

It follows from theoretical considerations that two ampholytes, the isoelectric points of which are distributed on both sides of the pH of the pure solvent, can be fully separated by stationary electrophoresis[11]. This situation occurs necessarily during every focusing of ampholytic carriers with the consequence that current during focusing drops to a certain limiting value which is determined by the ratio of diffusion rate and focusing of two ampholytes, the isoelectric points of which are separated by neutral pH.

It thus appears unnecessary to prolong the focusing process beyond the point when the limiting current is reached. Experience shows that a sharp zone *e.g.* of haemoglobin in a liquid column stabilized with sucrose is produced within 3–4 h while the limiting value of the current was reached in these experiments only after some 24 h of focusing. Too long focusing runs should be avoided also because the properties of the peptone gradient deteriorate in that its alkaline part gradually becomes steeper as focusing is prolonged. A similar observation was made with the polyacrylamide columns using Ampholines[11].

An example of separation of Hb A and Hb F (Fig. 5*) shows that the resolving power of the bactopeptone carrier is at least 0·1 pH unit. Undoubtedly, the resolving power of bactopeptone can be further increased by isoelectric fractionation and by using fractions with a smaller pH range. Technically speaking, bactopeptone is a satisfactory ampholyte carrier. We did not observe the formation of coloured zones or of precipitates in a stabilized liquid column. For separation of isolated proteins from peptides, gel filtration is a speedier technique than dialysis. The only drawback of bactopeptone lies in the fact that optical monitoring during automatic fraction collection is imposible.

Isoelectric fractionation of proteins on polyacrylamide columns appears to be an unusually sensitive aid for discovering a great number of components in protein mixtures. Its application to the determination of the isoelectric point of proteins is subject to some limitations: the pH of the extract of polyacrylamide gel sections is not identical with the pH of the section centre along the longitudinal axis; the effect of dilution during the extraction on the pH cannot be determined; protein focusing deforms the pH gradient estimated in a column of pure carrier.

In a comparison of the focusing patterns in columns with Ampholines and with bactopeptone one cannot expect identity either in localization or in mutual spatial

* See insert facing p. 326.

position of the zones. Hence one of the carriers cannot serve as reference for the other and the results obtained are to be treated separately. Our experience with bactopeptone in a polyacrylamide gel indicates that in dilute haemolyzates of human erythrocytes we often find haemoglobin plus 8 other zones. This suggests not only the outstanding capability of the focusing technique but also the high resolution power of the bactopeptone carrier.

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Translated by A. Kotyk.