SYNTHETIC AMPHOLYTES FOR THE ISOELECTRIC FOCUSING OF PROTEINS

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SUMMARY

Products of the copolymerization of acrylic acid with oligoethylene oligoamines were used to establish natural pH gradients for the isoelectric fractionation of protein mixtures by polyacrylamide gel electrophoresis. The unfractionated synthesized ampholytes were compared with the commercially available ampholytes with respect to the number of ampholyte components, the pH gradient and the resolution of protein mixtures. The performance of the ampholytes prepared employing the more highly substituted ethylene amines was found to be comparable to that of the commercial ampholytes over the pH range 4 to 8.

INTRODUCTION

The technique of isoelectric focusing in natural pH gradients (Vesterberg and Svensson, 1966) is considered to be one of the most sensitive tools for the detection of heterogeneity in proteins and other charged macromolecules (Vesterberg, 1971). Its use employing either sucrose-stabilized density gradients or polyacrylamide gels, requires a mixture of low molecular weight ampholytes with evenly spaced isoelectric points differing by 0.1 pH unit or less. The ampholytes should also possess sufficient buffering capacity and a conductance large enough to be able to form a stable natural pH gradient (Svensson, 1962).

Finding ourselved unsatisfied with the performance of commercially available ampholytes and their reproducibility, we decided to synthesize ampholytes by the copolymerization of acrylic acid with oligoethylene oligoamines. We report below some of the preliminary results of the iso-

electric focusing of protein mixtures using our ampholytes and commercially available products.

MATERIALS AND METHODS

Acrylic acid (Aldrich Chem. Co.) was distilled under vacuum immediately before use in order to remove the polymerization inhibitor (200 ppm p-methoxyphenol). The triethylenetetramine (TETA) and tetraethylenepentamine (TEPA) (Aldrich Chem. Co.) and the pentaethylenehexamine (PEHA) (Union Carbide Corp.) were distilled under vacuum before use.

Acrylamide and bis-acrylamide (Eastman Kodak Co.) were twice recrystallized from chloroform and acetone, respectively (Loening, 1967). Ampholines (pH 3-10) were obtained from LKB Produkter, Sweden (Lots 45, 48 and 50). The β-lactoglobulin (Pentex, Lot 34) and horse myoglobin (Nutritional Biochemicals Corp., #2340) were used without purification. All other materials were reagent grade.

Synthesis of Ampholytes. The general procedure followed was similar to that employed by Vesterberg (1969). All reactions were run in an inert atmosphere of nitrogen. To a stirred solution of 0.15 moles of the polyamine in 35 ml of water, acrylic acid was added dropwise over 45 to 60 minutes to provide the desired nitrogen/carboxyl ratio. After the addition of the acrylic acid was complete, the reaction temperature was adjusted to 70° C and the solution stirred overnight (16-20 hours). The reaction mixture was then cooled to room temperature and sufficient distilled, deionized water added to make a 40 %w/w solution of the ampholyte mixture. On a few occasions the reaction product was tested for unreacted acrylic acid by titrating an aliquot with $KMnO_4$. All samples tested showed that more than 99% of the acrylic acid had reacted. The ampholytes were stored in brown bottles in a refrigerator. The absorption of the ampholytes in the 250-400 nm range was reduced by repeated (3-4 times) treatment of a 15-20% w/w aqueous solution of the ampholytes with activated charcoal at 80-90° C.

Acrylamide Gel Electrophoresis. Gels containing 7-10% w/w total acrylamide, 2-4% cross-linking and 2% w/v of ampholytes were polymerized with 0.5-2% ammonium persulfate (final conc. in gels 0.028%-0.22% w/v) in pyrex tubes (10 cm x 0.4 cm). All gels were preelectrophoresed for 20 min. prior to the application of the samples to remove excess persulfate. Electrofocusing was performed at ca. 10° C in a Buchler Polyanalyst apparatus,

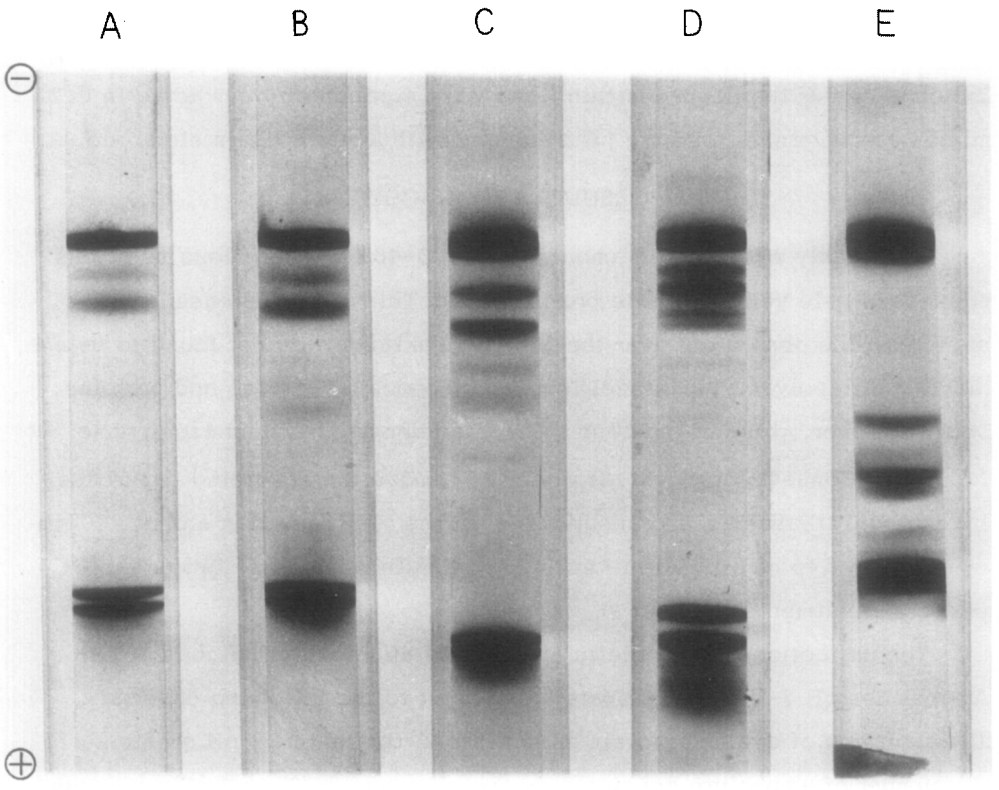

Figure 1. Polyacrylamide gel isoelectric focusing of a mixture of myoglobin and β-lactoglobulin. The gels were aligned with respect to the principal myoglobin band. A-LKB Ampholine (batch #45); B-LKB Ampholine (batch #48); C-PEHA Ampholyte (#6); D-PEHA Ampholyte (#6 charcoal treated); E-TEPA Ampholyte (#5).

at a constant voltage of 150 V for a duration of about 16 hrs. The cathode electrolyte used was 4% (v/v) monoethanolamine and the anode fluid was 2% (v/v) H_3PO_4. Samples were applied on the alkaline (top) side of the gels. The gels containing protein were fixed in 12.5% trichloroacetic acid solution and stained with Coomassie Blue (Spencer and King, 1971) and destained by diffusion. The natural pH gradients established upon electrophoresis were determined by measurement of the pH of 0.5 cm thick slices of the gel placed in 0.5 ml of distilled, deionized water.

Detection of Ampholyte Patterns. Sephadex G-75 thin layer isoelectric focusing (Radola, 1969) with 2% w/w ampholyte solutions was carried out at 5° C, employing a constant voltage of 300 v for 6 hrs. and 400 v for the

last hour. The caramelization patterns of the focused ampholytes were obtained as described by Felgenhauer and Pak (1973). For pH measurements, 2xl cm areas of the Sephadex thin layer were scraped off, extracted in 0.2 ml of water overnight and the pH measured with a micro glass electrode.

RESULTS AND DISCUSSION

The freshly synthesized ampholytes in 10-40% w/w aqueous solutions possessed pale yellow to pale brown colors. This color increased upon standing. The absorption over the 250-400 nm range was not found to be a hindrance in polyacrylamide gel isoelectric focusing of dyes and proteins. This, however, was not the case in sucrose density gradient isoelectric focusing. Consequently, we attempted to remove the absorbing impurities by charcoal treatment. The absorbances in the visible region and at 280 nm were reduced to ca. 0.1 for a 1% w/v solution in water after two or three charcoal treatments.

The unfractionated synthetic ampholytes and several batches of LKB Ampholines pH 3-10 were compared according to the following criteria: (1) resolution of dye and protein mixtures, (2) the (natural) pH gradient, (3) number of ampholyte species over the pH range 3-10, and (4) evenness of distribution and concentration profiles of ampholyte species.

Horse myoglobin and β-lactoglobulin were used to evaluate the resolution achieved with various ampholyte preparations. The A and B forms of the latter provide a good marker at about pH 5 (Kaplan and Foster, 1971). Myoglobin is known to provide a pattern of six (Vesterberg, 1967) or seven (Van den Oord et al., 1969) bands on isoelectric focusing over the pH range 6.5 to 7.5. Representative polyacrylamide gel isoelectric focusing patterns of a mixture of horse myoglobin and β-lactoglobulin obtained with various ampholytes are shown in Figure 1. The pH profiles of synthetic ampholyte preparations obtained from the caramelization patterns (Felgenhauer and Pak, 1973) are shown in Figure 2. The locations of individual ampholytes are indicated by arrows. We found that useful ampholyte mixtures were obtained only upon copolymerization of acrylic acid with PEHA. In the pH region 4 to 8 the resolution of the synthetic material is comparable to that of the commercial product. The synthetic ampholyte mixture forms a smooth natural gradient (Figure 2). The number of individual ampholytes observed

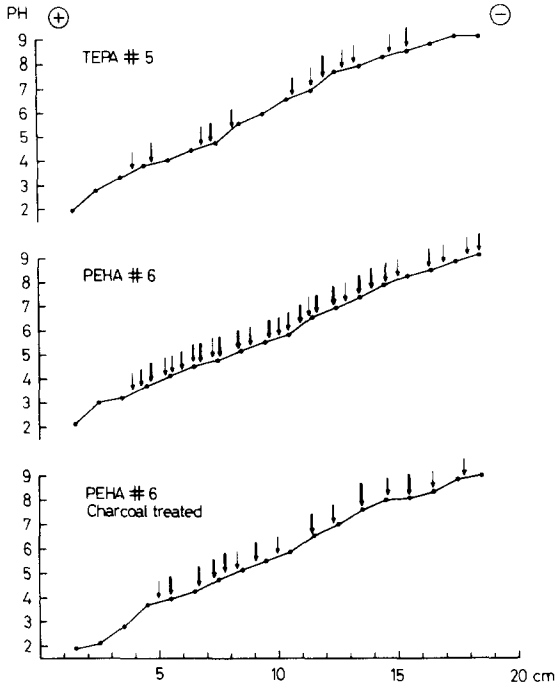

Figure 2. The pH profiles of three synthetic ampholyte preparations obtained from thin-layer isoelectric focusing.

in the products of the copolymerization of acrylic acid with PEHA is about the same as in LKB pH 3-10 Ampholines.

Different ratios of oligoamine to acrylic acid were used in the copolymerization. It was found that the most satisfactory ampholytes were obtained with nitrogen/carboxyl ratios of about 2:1. The resolution obtained with synthetic ampholytes made with TETA and TEPA was poor (Figure 1) due primarily to the small number of individual ampholytes (Figure 2). Gas chromatographic and mass spectrometric analyses of distilled, commercially available TETA, TEPA and PEHA (Bergstedt and Widmark, 1970) have shown these to possess 7, 12 and 20 fractions, respectively, corresponding closely to the expected number of structural isomers present. Thus the principal reason for the success of PEHA copolymerizations with acrylic acid is probably the large number of structurally different isomers present initially in the amine, which on copolymerization with acrylic acid, produce a large number of ampholytes with closely spaced pI's. The total number of individual species in PEHA ampholytes varied from 20 to 30 (Figure 2) and was about twice

as great as in TEPA ampholytes. Treatment of PEHA ampholytes with charcoal reduced substantially the number of individual species (Figure 2), suggesting that the color is due to the presence of nitrogen heterocyclic structures. Work is in progress on the improvement of PEHA ampholytes, the synthesis of other ampholytes and on their characterization.

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