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Influence of additives on resolution and focusing efficiency in free-flow isoelectric focusing

Gerhard Küllertz*, Gunter Fischer

Max-Planck-Gesellschaft zur Förderung der Wissenschaften eV, Arbeitsgruppe "Enzymologie der Peptidbindung", Weinbergweg 16a, 06120 Halle/Saale, Germany

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Abstract

In theory, the resolution of isoelectric focusing (IEF) depends directly on the electric field strength. However, at higher electric field strengths with free-flow IEF, focusing of proteins is impaired by electrodispersive effects. These effects cause a widening of protein peaks sharply focused at lower voltage. This was dependent on the electric field strength and thus reduced the focusing efficiency and resolution. Using 4-nitroaniline as a visible marker substance, the influence of concentration and molecular mass of various additives on the electrodispersive effects as a function of electric field strength was investigated. The efficiency to reduce electrodispersive effects was determined by various properties of the additives. However, the molecular mass of the additives only marginally influenced electrodispersive effects. In the most favourable case, the resolution R for the separation of bovine serum albumin and haemoglobin applied as a standard mixture increased from $R = 0.4$ without additive to $R = 3.4$ with 2% saccharose and 0.3% Servalyte 3–10 as ampholyte. Improvements in the resolution for the separation for the standard mixture were independent of the ampholyte used (Bio-Lyte, Ampholine, Servalyte).

1. Introduction

Isoelectric focusing (IEF) is a kind of electrophoresis performed in a pH gradient where amphoteric molecules are separated according to their individual isoelectric points (pI). This isoelectric pH value is a characteristic physicochemical property of each protein and is affected by the overall composition of the protein and the properties of the medium. The history of IEF is relatively short and dates back to the early 1960s [1,2].

Four techniques are useful for laboratory-scale preparative electrofocusing: (1) IEF in order on granular beds [3], (2) IEF in order on a separation chamber that is divided into compartments [4,5], (3) a recycling isoelectric focusing method [6,7] and (4) a continuous free-flow isoelectric focusing method [8].

IEF is a technique allowing enrichment of zwitterionic molecules with respect to their spatial pH distribution depending on a uniform d.c. voltage. The production of this stationary, steady-state sample distribution is time consuming because the electrophoretic velocity tends towards zero when the migration of the proteins in the pH gradient carries them towards their pI. The steady state is established where the electrokinetic transport of protein into the focusing zone is exactly balanced by the diffusion out of

* Corresponding author.

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this zone. For IEF in a column, experimental times of at least 18–24 h [9] and, in membrane-defined subcompartments [4,5], focusing times of 1.5–5 h are usually satisfactory. Free-flow IEF apparatus requires a transition time of about 3 h using a recycling system [10] and less than 20 min [8] as a continuous device.

The differential equation describing the equilibrium between the electrophoretic and diffusional mass transport was derived by Svensson [2]:

$$\frac{dC_i}{dx} = \frac{C_i \mu_i E}{D} \quad (1)$$

where C_i is the concentration of component i, μ_i is its mobility, E is the electric field strength, D is the diffusion coefficient of the solution and dC_i/dx is the concentration gradient. This equation shows that both increasing the mobility and electric field strength and reducing the diffusion will lead to an increase in the narrowing of the focusing zone. Because the viscosity of the solution has an opposite influence on the diffusion and the mobility of proteins, it is difficult to predict the effect of the viscosity on the focusing process.

In addition, the focusing of proteins will be affected by electrodispersive effects (EDE) depending on the electric field strength. Such effects can be seen as a broadening of otherwise sharply focused protein peaks depending on the electric field strength [8,10]. To manipulate the EDE with additives, extensive experiments have been carried out on capillary electrophoresis [11]. Based on these data, we determined the influence of different transport phenomena on the sample dispersion in continuous free-flow IEF to improve the resolution of this method.

2. Experimental

2.1. Standards for calibration of native IEF

Haemoglobin (human; pI = 7.0) was obtained from Boehringer (Mannheim, Germany). Bovine serum albumin (pI = 5.2), ovalbumin (chicken egg albumin; pI = 4.6) and 4-nitroaniline (pK_a = 1.3) were obtained from Sigma (Deisenhofen, Germany).

2.2. Solutions for IEF

Solutions of Servalyte (Serva, Heidelberg, Germany), pH range 3–10, 4–7, 4–5 or 5–8, solutions of Ampholine (Pharmacia, Uppsala, Sweden), pH range 5–8, and solutions of Bio-Lyte (Bio-Rad, Hercules, CA, USA), pH range 5–8 with concentrations of 0.1–0.4% (w/v), were prepared with deionized (Milli-Q Plus; Millipore, Eschborn, Germany) and CO_2-free water. The catholyte was 0.05 M sodium hydroxide and the anolyte was 0.5 M orthophosphoric acid. Both solutions were used as membrane rinse solutions at their appropriate position.

2.3. Additives to manipulate the focusing efficiency

Different concentrations of the following additives were used to manipulate the focusing efficiency and resolution: glycerol from Roth (Karlsruhe, Germany), polyethylene glycol (PEG) with a molecular mass of 10^6 (PEG 1-kDa) from Merck–Schuchardt (Hohenbrunn, Germany), PEG of molecular mass $6 \cdot 10^3$ (PEG 600-kDa) from Serva, saccharose, sorbose and polyvinylpyrrolidone (PVP) with a molecular mass of $3.6 \cdot 10^5$ (PVP 360-kDa) from Serva, PVP with a molecular mass of $2.5 \cdot 10^4$ (PVP 25-kDa) from Merck (Darmstadt, Germany), hydroxypropylmethylcellulose (HPMC) from Aldrich (Milwaukee, WI, USA) and dextrans with average molecular masses of 1000, $4 \cdot 10^4$, $7 \cdot 10^4$, $11 \cdot 10^4$ and $5 \cdot 10^5$ from Pfeifer & Langen Pharma (Dormagen, Germany).

2.4. Instruments

The principle of the preparative continuous isoelectrofocusing apparatus (Dr. Weber, Ismaning, Germany) and the equipment belonging to it and used here were described in detail previously [8].

The specific conductivity of solutions was determined in a conductivity cell with a cell con-

stant of 0.998 cm^{-1} connected to a Type LF530 precision conductivity meter (Wissenschaftliche-Technische Werkstätten, Weilheim, Germany).

Spectrophotometric measurements were carried out with a Type MR7000 microtitre plate reader (Dynatech, Chantilly, VA, USA).

The ionization data were calculated from titration curves according to Cohn et al. [12]. Titration curves were taken with a Type VIT90-video titrator (Radiometer, Copenhagen, Denmark).

2.5. Determination of protein, dextran and 4-nitroaniline concentrations

Protein concentrations were determined by the Bradford procedure [13] using bovine serum albumin as a standard at 625 nm. Dextran concentrations were confirmed with an anthrone method at 625 nm and dextran as standard [14]. 4-Nitroaniline concentration was measured utilizing the Lambert–Beer law at 390 nm (ε = 11 400 l mol^{-1} cm^{-1}).

2.6. Resolution and focusing efficiency

A method for specifying resolution (R) in IEF is in terms of the peak distance (Δx) in cm (Fig. 1), fraction numbers or pH values of the peak maximum of adjacent peaks. If the two detected peaks (a and b) have a symmetrical shape and Gaussian profiles with a baseline peak width of 4σ, the following equation will be used for the calculation of resolution in IEF experiments:

$$R = |\Delta x|/2(\sigma_a + \sigma_b) \quad (2)$$

The peak width (Fig. 1) at the peak half-height ($w_{1/2}$) is about 2.35σ for symmetrical peaks. Therefore, for practical purposes the focusing resolution of two symmetrical peaks can be defined as

$$R = |\Delta x|/0.85(w_{1/2a} + w_{1/2b}) \quad (3)$$

Peaks recorded in IEF are not always Gaussian. In a number of cases, especially with non-linear pH gradients, the bands are asymmetric. The resolution of such peaks was calculated using the equation

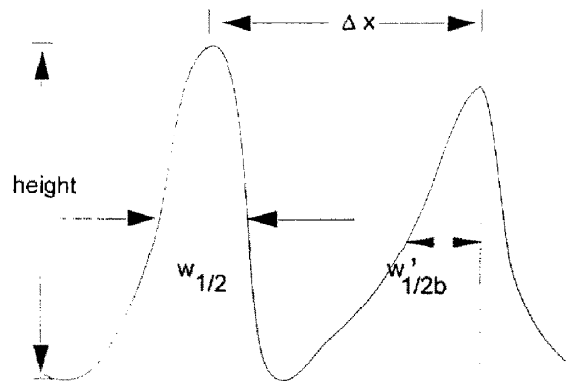


Fig. 1. Schematic representation of two peaks in IEF to characterize separation parameters: $w_{1/2}$ = peak width at half-height; $w'_{1/2}$ = segment of the baseline $w_{1/2}$ as intersection with the vertical from the peak maximum and $w_{1/2}$; Δx = distance in cm, fraction numbers or pH values of the peak maximum between adjacent peaks.

$$R = |\Delta x|/1.71(w'_{1/2a} + w'_{1/2b}) \quad (4)$$

The segment of the baseline $w'_{1/2}$ (Fig. 1) is defined by its intersection with the vertical from the peak maximum and $w_{1/2}$. For the calculation of R, two adjacent segments ($w'_{1/2a} + w'_{1/2b}$) of non-Gaussian peaks were used. For a quantitative description of the focusing efficiency (F_{eff}) of a single protein peak, we used the equation

$$F_{eff} = 1/\sigma^2 \quad (5)$$

or for practical purposes

$$F_{eff} = 5.54(1/\Delta pH_{1/2})^2 \quad (6)$$

where $\Delta pH_{1/2}$ is the pH difference of $w_{1/2}$ [8]. Because non-linear pH gradients intrinsically distort the Gaussian form of protein peaks, non-linear pH gradients were corrected by computational interconversion to a linear form before F_{eff} was calculated [8].

2.7. Determination of the apparent diffusion coefficient

The diffusion coefficient (D) of proteins was determined using the Gaussian spreading by

diffusion processes of the respective protein in the IEF separation chamber in the absence of an electric field. Based on Einstein's equation ($\sigma^2 = 2Dt$) we calculated D using the equation

$$D = \frac{(\Delta w_{1/2})^2}{3.07\Delta t} \quad (7)$$

where $\Delta w_{1/2}$ is the difference in the peak half-width at different transit times (Δt) of the sample.

3. Results and discussion

3.1. Influence of electrodispersive effects on focusing efficiency (F_{eff})

The negative influence of electrodispersive effects (EDE) on the resolution in IEF has been described by several workers [10,11,15,16] based on investigations with different electrophoretic devices. Fawcett [15] and others have explored the use of free-flow electrophoretic instruments for isoelectric focusing, but the results were described as "rather disappointing". Increasing the voltage resulted in "feathering", a breakdown of the flow lanes into feather-like structures.

Using colored marker substances such as haemoglobin, the line shape of these focused substances can be observed visually in the separation chamber. In contrast to the optimum electric field strength, at higher voltages broadening of substance distribution occurs [8]. The influence of a stepwise increase in electric field strength (from 50 to 200 V/cm^{-1}) on the F_{eff} of human haemoglobin is shown in Fig. 2. Between 100 and 110 V/cm^{-1} an optimum F_{eff} was detected whereas at higher electric field strengths F_{eff} decreased drastically. This optimum is caused by the concerted action of processes that mainly consist of electrophoresis, electroosmosis, streaming potential and sedimentation potential [17]. The total variance σ_T^2 ($\sigma_T^2 = 1/F_{eff}$) of these effects results from the sum of the contributing variances:

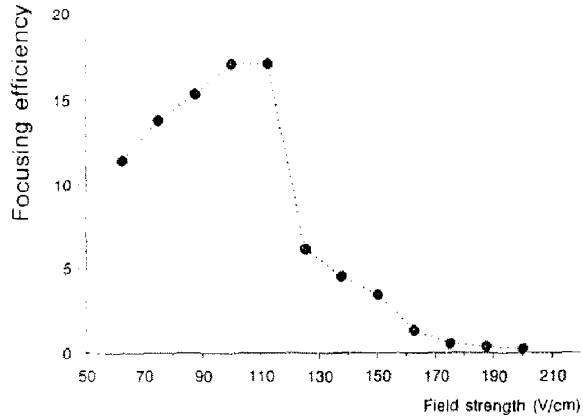


Fig. 2. F_{eff} of human haemoglobin as a function of the electric field strength in IEF. Experimental conditions of IEF: temperature of chamber, 4°C; medium, 0.3% (v/v) Servalyte 3–10/4% (w/v) glycerol; transit time, 8 min.

$$\sigma_T^2 = \sigma_{dif}^2 + \sigma_{conv}^2 + \sigma_{ads}^2 + \sigma_{eld}^2 + \sigma_{oth}^2 \quad (8)$$

where the subscripts refer to diffusion, convection by temperature gradients, adsorption on the walls of the electrophoresis chamber, electrodispersion and other effects, respectively. Electrodispersive effects can be caused by the potential gradient of the electric field in IEF (σ_{ief}^2), by conductivity gradients (σ_{cond}^2) and by the electroosmotic potential (σ_{eos}^2).

In relation to the electric field we can classify the variances into three groups. There are variances that are mainly independent of field strength (σ_{dif}^2 and σ_{ads}^2) and also variances that increase with increasing electric field strength (σ_{conv}^2, σ_{cond}^2 and σ_{eos}^2) and that will decrease with the electric field strength (σ_{ief}^2). The dependence of F_{eff} on the electric field strength in Fig. 2 is the overall result of these effects.

In order to measure exclusively electrokinetic phenomena, uncharged marker molecules may be helpful. Their migrations depend only on the passive transport by the focusing medium. Therefore, we investigated the effect of EDE using 4-nitroaniline as indicator substance. With a pK_a value of approximately 1.3 [18] this substance occurs uncharged in a solution with a

pH gradient of 3–10 and also at the starting pH of the separation medium (ca. 6.5). The 4-nitroaniline solution was introduced as a narrow stream in the middle of the bottom of the electrophoresis chamber. The widening of the 4-nitroaniline lane after applying an electric voltage was evaluated as a measure of EDE.

At a field strength above 120 V cm^{-1} there was a sharp decline for the F_{eff} of haemoglobin (Fig. 2). Concomitantly, an increase in the peak width at half-height of 4-nitroaniline (Fig. 3) occurred. In addition, a non-linear change of the electrical resistance as a result of increasing electric field strength enhances the value of EDE (Fig. 3). According to Ohm's law, the plot of the applied field strength versus the resulting current should be linear. Deviations from linearity described for capillary zone electrophoresis probably arise from enhanced heat generation at higher potential differences [19]. However, temperature measurements (standard error ca. 0.1°C) in the 96-channel outlet port during IEF showed no significant temperature drift depending on the electric field strength.

The peak broadening of the 4-nitroaniline concentration is directed to both electrodes. The drift to the anodic side is half as much as the drift to the cathode (Fig. 3). A similar effect can

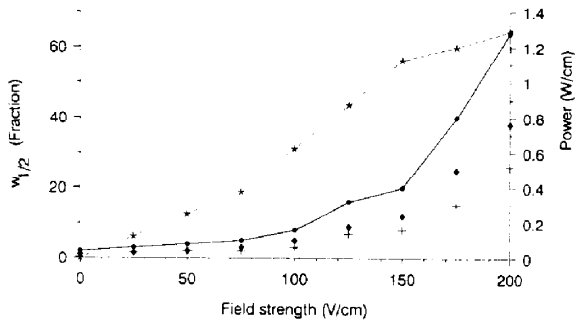


Fig. 3. Influence of the electric field strength on electric power and $w_{1/2}$ of a saturated 4-nitroaniline solution. Experimental conditions of IEF: temperature of chamber, 5°C; medium, 0.2% (v/v) Servalyte 3–10/3% (w/v) glycerol; transit time, 8 min. ● = Peak width at half-height; * = power (W cm^{-1}); + = $w'_{1/2}$ towards the anode; ◆ = $w'_{1/2}$ towards the cathode.

be obtained by investigating the EOF in recycling free-flow IEF [10].

3.2. Influence of additives on electroosmotic flow (EOF)

To control electroosmotic flow, surface modification has been used to manipulate the EOF in capillary zone electrophoresis [11,20]. It was shown that simple manipulation of the ionic composition of the buffer can reduce the EOF by decreasing the double-layer thickness [21]. The flow-rate in capillary zone electrophoresis was found to be inversely proportional to ionic strength [11]. The addition of organic solvents can dramatically influence the electroosmotic flow [11]. However, salts and organic solvents as additives may not be suitable for the separation of native proteins with IEF, because organic solvents lead to the formation of protein aggregates. On the other hand, ionic substances increase the conductivity of the focusing solution and may reduce the resolution by enhanced thermal convection.

It was recently established that the addition of amphiphilic copolymers or oligomers that contain both hydrophobic and hydrophilic groups can efficiently control the IEF in capillary electrophoresis [11,20]. These investigations also suggest [20] that the degree of reduction in EOF is proportional to the size of the hydrophilic portion of the surfactant. In a capillary the EOF could be adjusted to any desired value by adsorbing surfactants and polymers of various size on the capillary wall.

By adding to the flow solution one of the surfactants or polymers glycerol, sorbose, saccharose, polyvinylpyrrolidone (PVP), hydroxypropylmethylcellulose (HPMC), polyethylene glycol (PEG) and dextran, the influence on the resolution and focusing efficiency was tested with preparative continuous IEF. Coatings of the chamber walls were applied by passing about 50 chamber volumes of surfactant solution through the focusing chamber. Subsequent separations were carried out using ampholyte solutions containing the same concentration of surfactant.

Additives can have different effects on IEF. First, they modulate the EOF and reduce the adsorption of samples in the separation chamber. Second, an increase in the viscosity and/or density of the focusing solution is obtained. Hence impaired mobility of charged substances in an electric field may result. Because free-flow IEF has a limited focusing time, such reduced mobility may result in a poor pH gradient. The quality of the pH gradient in the IEF equipment used can be evaluated after measurement of the pH in each of the 96 fractions, which are continuously produced by the sample outlet connector [8]. With Servalyte 3–10 as ampholyte and sufficient time to create a linear pH gradient at an optimum electric field strength, a pH difference between fraction 20 (ca. pH 4) and fraction 76 (ca. pH 9) of about 5 pH units can be expected.

Fig. 4 shows that the quality of the pH gradient, expressed as $\Delta pH_{20/76}$ (pH difference between fractions 20 and 76), becomes worse if the concentration of glycerol is increased from 1 to 30% (w/v). In parallel, the value of $w_{1/2}$ of 4-nitroaniline as EDE marker substance is decreased. Increasing the electric field strength from 125 to 200 V cm^{-1} exerts an opposite effect on both EDE and the quality of the pH gradient (Fig. 4).

Additives that provide both an excellent pH gradient and a small EDE are 1% (w/v) PVP (10-kDa), 0.002% (w/v) HPMC, 2% (w/v) saccharose, 0.1% (w/v) dextran (1-kDa) and PEG. Other substances such as glycerol, sorbose, 2% (w/v) PVP (10-kDa), PVP (360-kDa) and dextran with a molecular mass of $4 \cdot 10^4$ and higher are apparently less suitable. Adsorption of polymers on surfaces is described as a loop-and-train mechanism [20]. As the molecular mass of the additives increases, the adsorbed coating becomes thicker and more viscous. In agreement with this finding, EOF was inversely related to the molecular mass of the coating polymer in capillary isoelectric focusing [20]. In our experiments however, we found that $w_{1/2}$ does not depend exclusively on molecular mass of the coating polymer. Whereas PVP and PEG decreased the EDE with increasing molecular mass, six different dextrans with molecular masses from 1000 to $5 \cdot 10^5$ did not show this size effect.

As can be seen in Fig. 4, large differences of EDE in IEF are achieved almost exclusively by a different chemical structure and are only margi-

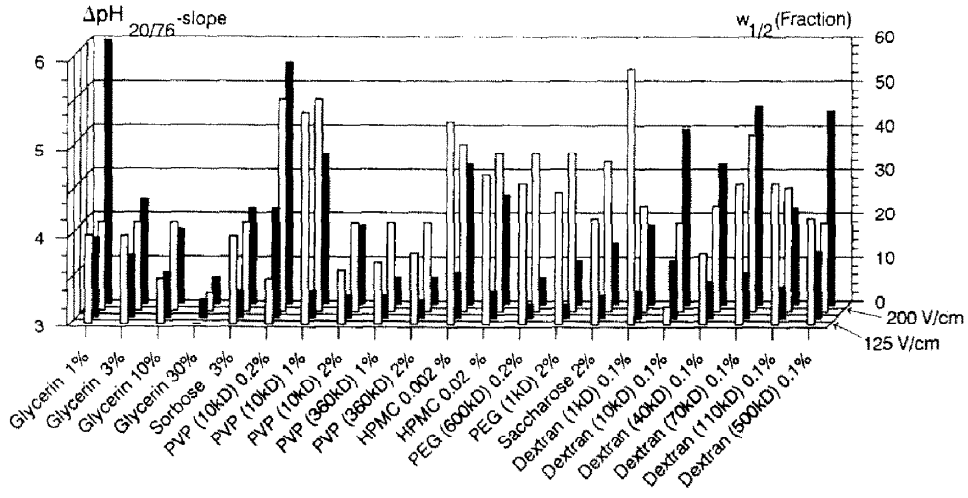
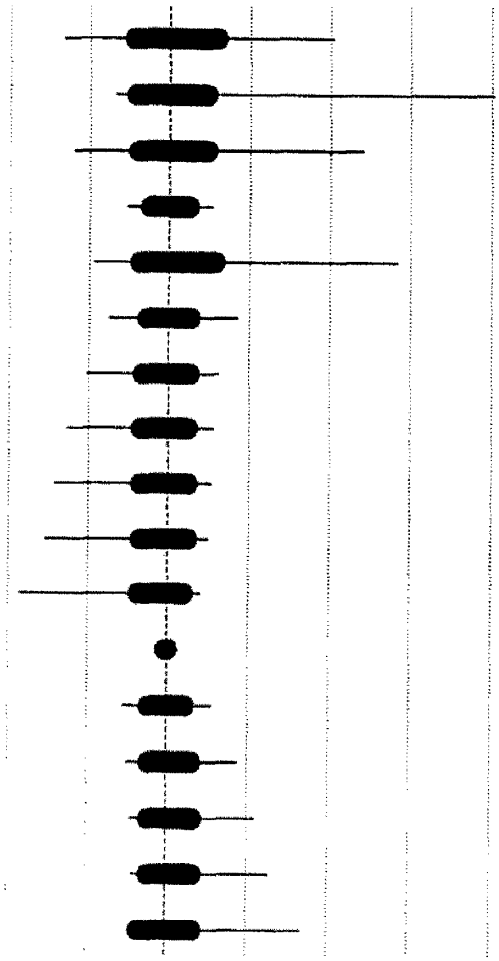


Fig. 4. Influence of additives on the peak width at half-height of a saturated 4-nitroaniline solution and the pH slope between fractions 20 and 76 ($\Delta pH_{20/76}$) as a function of electric field strength. Experimental conditions of IEF: temperature of chamber, 5°C; medium, 0.4% (v/v) Servalyte 3–10; transit time, 10 min. □ = Peak width at half-height; ■ = $\Delta pH_{20/76}$ slope.

Table 1
Isoelectric points and charges

Protein, amino acid or polypeptide	p*I*	Net charge (proton units)		Charge/radius coefficients at different pH[b] (proton units/cm)
		±0.3	±1.0	−0.4 0 0.4 0.8 1.2 1.6
Bovine serum albumin	5.7	9	39	
Bovine serum albumin[a]	4.8	6	41	
Human serum albumin	5.9	9	33	
Human haemoglobin[a]	7.0	4.5	23	
Ovalbumin	5.3	7.5	30	
Asparagine (D)	3.6	0.55	1.5	
NH_2-D_2-COOH	3.4	0.76	2.0	
NH_2-D_3-COOH	3.3	0.88	2.7	
NH_2-D_4-COOH	3.2	0.97	3.2	
NH_2-D_5-COOH	3.1	1.0	3.5	
NH_2-D_{10}-COOH	2.9	1.2	4.9	
Arginine (R)	10.1	0.04	0.2	
NH_2-R_2-COOH	12.0	0.70	1.6	
NH_2-R_3-COOH	12.3	0.90	2.2	
NH_2-R_4-COOH	12.5	1.02	2.9	
NH_2-R_5-COOH	12.6	1.11	3.4	
NH_2-R_{10}-COOH	13.0	1.7	4.8	



Sequences were taken from Protein Identification-Resource (PIR), the international association of protein sequence banks. The isoelectric points and net charge differences were calculated according to Ref. [25]. Partial specific radii of proteins or peptides were calculated from the amino acid composition according to the volumes of the constituent amino acids [26]. Ionization data were calculated from titration curves and are in accordance with values for haemoglobin [12][a] and ionization data for bovine serum albumin [24][a] or values were calculated from the amino acid sequence of proteins in a range of 0.3 and 1 pH units around the p*I* (calculated or determined by IEF [b]) of selected proteins, peptides and amino acids and their charge/radius coefficients at this pH.

nally influenced by the molecular mass of polymers with a homologous structure. Earlier data [8] showed that EDE can be reduced by adding zwitterionic substances which exhibit very flat titration curves around their p*I* values (Table 1), e.g., N,N-bis(2-hydroxyethyl)glycine (bicine). The influence of increasing concentrations of bicine on the EDE is shown in Fig. 5. Higher concentrations of bicine clearly reduced the EDE. In comparison with monoionic additives, zwitterionic substances strongly affect the shape of the pH gradient [8]. Therefore, the application of these compounds will be limited to special separation problems in IEF.

3.3. Mobility of proteins in free-flow IEF

It is assumed that proteins require a longer time for focusing than ampholytes. Therefore, it was predicted that continuous-flow systems cannot be applied to IEF since the method lacks the long residence times required for protein focusing [10]. To test this prediction, we investigated the relationship between the transition time, R and F_{eff}. We investigated the separation of a sample containing bovine serum albumin and haemoglobin in IEF by stepwise changing the transition time. The results are summarized in Fig. 6. A transition time of 5–7 min at 150 V cm^{-1} gave a favourable resolution, whereas a significant deterioration below 5 min and a minor deterioration above 7 min were observed.

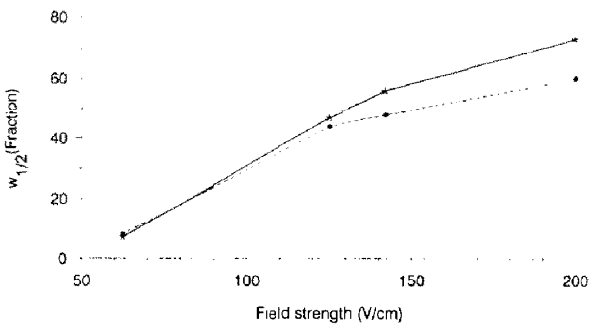


Fig. 5. Influence of bicine on the peak width at half-height of a saturated 4-nitroaniline solution as a function of electric voltage. Experimental conditions of IEF: temperature of chamber, 5°C; medium, 0.2% (v/v) Servalyte 3–10/4% (w/v) glycerol. ● = Peak width at half-height with 1% (w/v) bicine; ∗ = peak width at half-height without bicine.

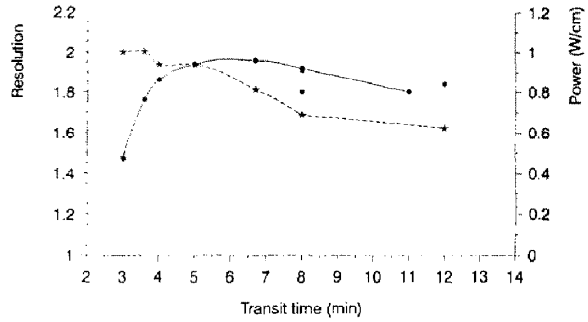


Fig. 6. Influence of transit time at a constant electric field strength on power and the peak resolution of bovine haemoglobin and bovine serum albumin. Experimental conditions: temperature of chamber, 5°C; medium, 0.2% (v/v) Servalyte 3–10/0.1% dextran 110-kDa; conductivity, 8.4 μS; electric field strength, 175 V cm^{-1}; sample solution, 1 mg ml^{-1} haemoglobin and 1 mg ml^{-1} bovine serum albumin in medium. ● = Resolution; ∗ = power (W cm^{-1}).

The short focusing times of these proteins can be explained in terms of the electric mobility of molecules that bear a relatively high net charge around their p*I* values. In detail, the electrophoretic mobility of proteins depends on the mobility slope ($d\mu/dpH$). A high value of this term results from the presence of many amino acids with dissociable groups having pK_a values close to the p*I* of the protein. Reasonable approximations can be obtained by calculation of net charge depending on the pH from the amino acid composition [22,23]. To estimate the mobility slope ($d\mu/dpH$) of different polypeptides, we calculated the net charge within 0.3 and 1.0 pH units around the p*I* value (Table 1) based on the amino acid sequence according to Ref. [25], and/or calculated the net charges from titration curves according to Ref. [12]. As expected, the net charge of the investigated proteins is higher than that of the investigated small zwitterionic substances. To calculate the electrophoretic mobilities (μ) we utilized the equation [27]

$$\mu = \frac{Q}{6\pi\eta a} \tag{9}$$

where Q is the effective charge of the ion, η the viscosity of the solution and $2\pi a$ the hydrodynamic circumference of the ion. Using the

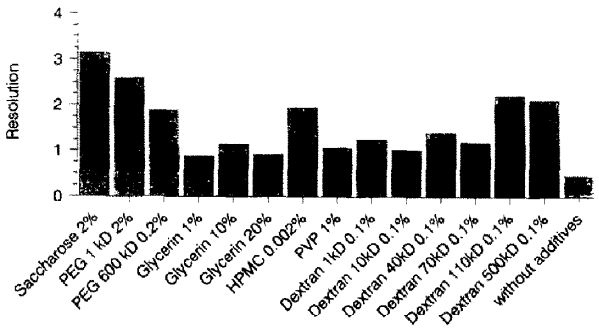


Fig. 7. Influence of additives on the resolution of a sample containing haemoglobin and bovine serum albumin. Experimental conditions: temperature of chamber, 4°C; medium, 0.2% Servalyte 3–10; sample solution, 1 mg ml^{-1} haemoglobin and 1 mg ml^{-1} bovine serum albumin in medium; electric field strength, 175 V cm^{-1}; transit time, 8 min. Concentrations of additives in % (w/v).

partial specific volumes of the constituent amino acids [26], the circumferences of proteins can be calculated. Commercial carrier ampholytes are mixed polymers of aliphatic amino and carboxylic acids of molecular mass ca. 300–1000 [16,28,29]. To calculate the magnitude of the differences between the electrophoretic mobilities of proteins and of carrier ampholytes we calculated the ratio of charge to radius (a) for proteins and polypeptides as a function of the pI of such molecules (Table 1). In contrast to electrophoretic methods in gels, where proteins are subject to sieving effects, in free solution the investigated proteins can migrate in an electrical field with velocities comparable to or even faster than those of small molecules.

3.4. *Influence of additives on resolution (R) of proteins*

To investigate the influence of various additives on the separation of proteins in free-flow IEF, we used a mixture of two proteins with different isoelectric points. Both proteins, human haemoglobin with a pI of ca. 7 and bovine serum albumin with a pI of ca. 5.2, were separated with a resolution of 0.8–3 depending on the nature and concentration of the additive. In contrast to the investigations with 4-nitroaniline as marker substance, a direct correlation between the molecular mass of the additives and

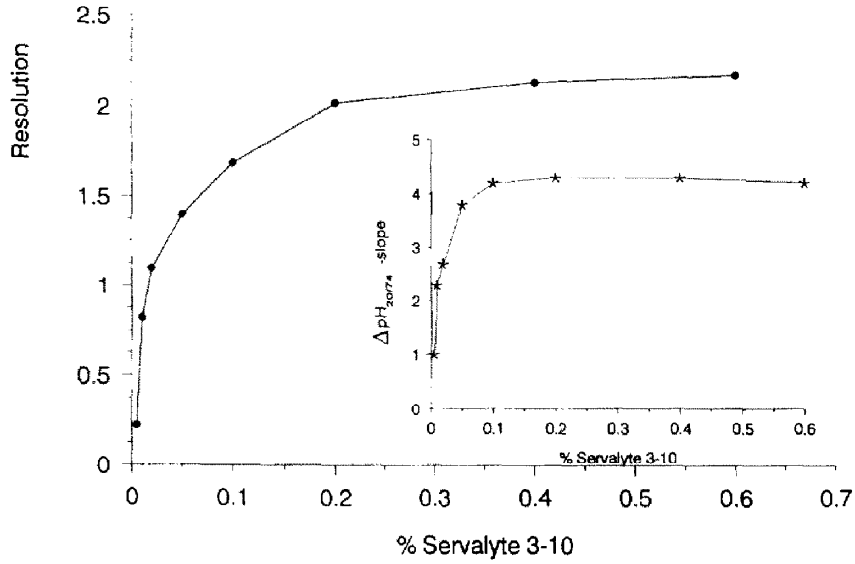


Fig. 8. Influence of ampholyte concentration on the resolution of a sample containing haemoglobin and bovine serum albumin and influence on the pH slope between fractions 20 and 76 (ΔpH_{20-76}). Experimental conditions: temperature of chamber, 5°C; medium, 0.1% (w/v) dextran 110-kDa; sample solution, 1 mg ml^{-1} haemoglobin and 1 mg ml^{-1} albumin in medium; electric field strength, 200 V cm^{-1}; transit time, 9 min. ● = Resolution; * = pH slope (ΔpH_{20-76}).

R was observed (Fig. 7). An improved separation of proteins by raising the molecular mass of the additives was also found in investigations with capillary isoelectric focusing [20]. It was suggested from the experiments that the EOF is influenced by additives [20]. In our investigations on 4-nitroaniline there is an obvious lack of evidence for a correlation of EOF with the molecular mass of additives. We conclude that there is a concerted action between electroosmotic effects and effects on the diffusion of the proteins by changes in the viscosity and density of the solution. However, less than a twofold change in EOF was achieved by varying the molecular mass of the polymer ranging from 1000 up to $5 \cdot 10^5$.

3.5. Effect of ampholyte concentration on resolution

An increase in the ampholyte concentration from 0 to 0.6% Servalyte 3–10 improved the resolution from 0 to about 2 for the separation of a sample containing human haemoglobin and bovine serum albumin (Fig. 8). There was no further substantial improvement in resolution when the ampholyte concentration became higher than 0.3%. The data in the inset in Fig. 8 show that a maximum pH slope is achieved even at a Servalyte concentration of 0.3%. Therefore, we suggest that a maximum pH slope should be applied for high resolution.

3.6. Effect of small ions on resolution

In contrast to zwitterionic substances, which focus at their intrinsic pI and have only a limited contribution to the current flux, unipolar ions will be transported in the direction of the counter-charged electrodes. Heat development, generated by passage of electric current, renders problems as it can cause non-uniform temperature gradients, local changes in viscosity and subsequent zone broadening [30]. Independent of this thermal convection, small ions affect the diffusion of large molecules. The small counter ions of proteins diffuse more rapidly than the oppositely charged macromolecules and increase the diffusion rate of the macroion [17]. Both an increase in thermal convection during IEF and an increase in diffusion at higher conductivity deteriorate R and F_{eff}. Our investigations (Fig. 9) shows that the resolution of the standard mixture containing haemoglobin and bovine serum albumin decreases with increasing conductivity. This is in agreement with experiments on IEF in gels. In the gel IEF method high salt concentrations cause band broadening [16].

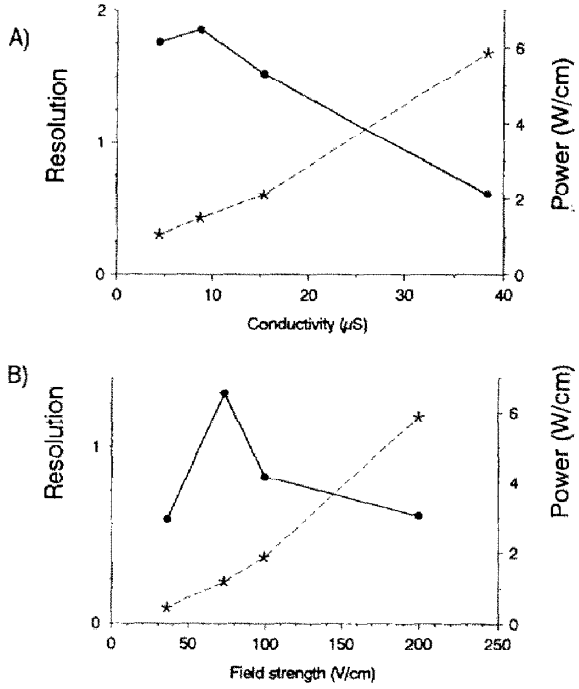


Fig. 9. (A) Effect of conductivity of the medium on the resolution of a sample containing haemoglobin and bovine serum albumin at an electric field strength of 200 V cm^{-1}. (B) Dependence of the resolution on the electric field strength (conductivity = 38.6 μS). Experimental conditions: temperature of chamber, 5°C; medium, 0.1% (w/v) dextran 110-kDa–0.2% (v/v) Servalyte 3–10 enriched with various amounts of sodium chloride; sample solution, 1 mg ml^{-1} haemoglobin and 1 mg ml^{-1} bovine serum albumin in medium; transit time, 9 min. ● = Resolution; * = power (W cm^{-1}).

3.7. Effect of different pH gradients on resolution

The resolution in IEF with respect to pI differences of focused proteins in IEF is given by [31,32]

$$\Delta pI = K\left[\frac{D(\mathrm{dpH/d}x)}{E(-\mathrm{d}\mu/\mathrm{dpH})}\right]^{0.5} \quad (10)$$

where K is the mean zone width (usually 4σ), D is the diffusion constant, E is the electric field strength, $\mathrm{d}\mu/\mathrm{dpH}$ is the mobility slope at the pI that depends on the charge of the protein around its pI value (Table 1) and $\mathrm{dpH/d}x$ is the pH gradient at this zone. As described by Eq. 10, high resolution should be obtained with a shallow profile of pH change. To check this relationship between R and the pH gradient in the continuous free-flow IEF, investigations on R and F_{eff} as a function of four different pH gradients produced with Servalyte 3–10, 5–8, 4–5 and 4–7 using a mixture of three different

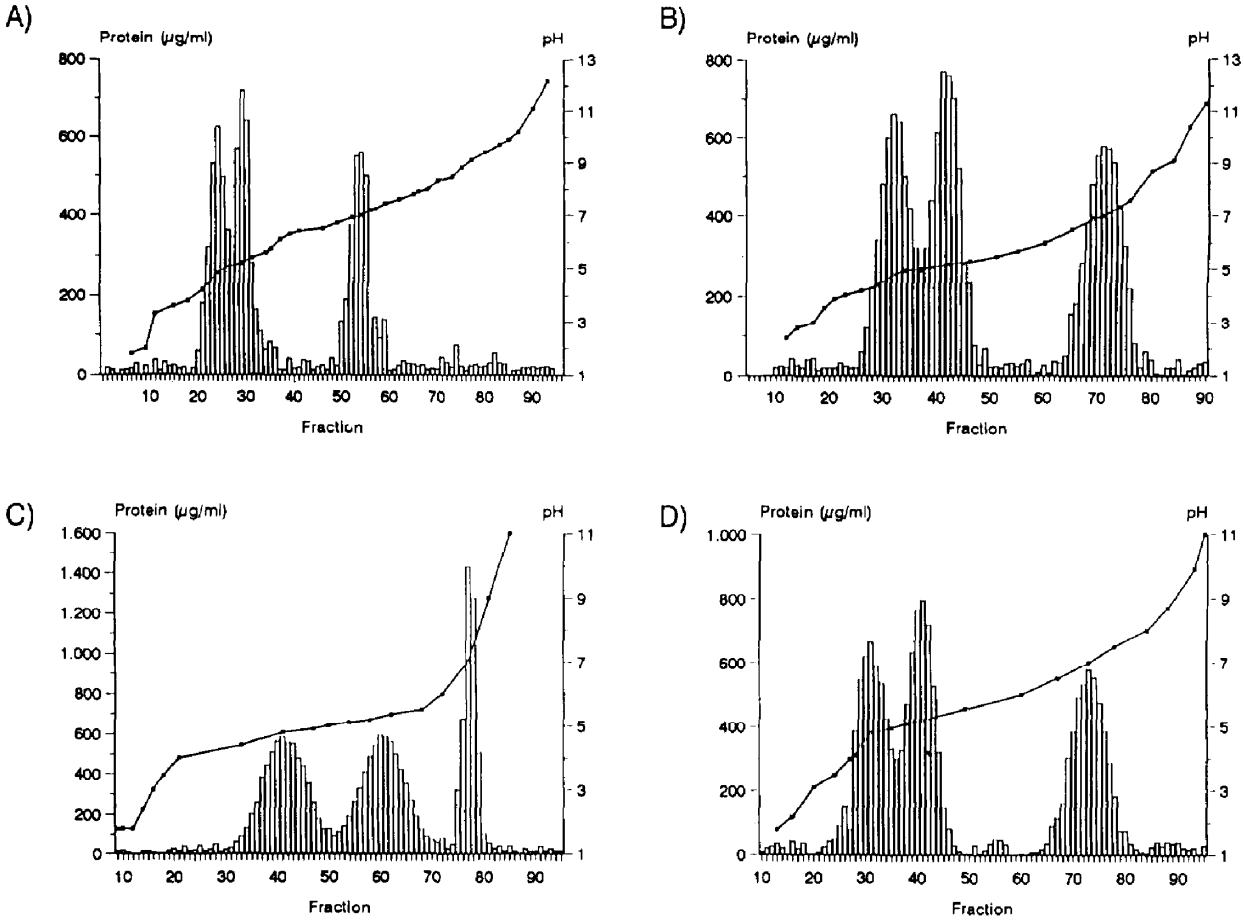


Fig. 10. Effect of different pH gradients on the separation of a sample containing ovalbumin, bovine serum albumin and haemoglobin. Experimental conditions: temperature of chamber, 5°C; medium, 3% (w/v) saccharose–0.35% (v/v) Servalyte; sample solution, 1 mg ml^{-1} ovalbumin, 1 mg ml^{-1} haemoglobin and 1 mg ml^{-1} bovine serum albumin in medium; electric field strength, 200 V cm^{-1}; transit time, 7 min. ● = pH gradient. (A) Servalyte 3–10; (B) Servalyte 4–5; (C) Servalyte 4–7; (D) Servalyte 5–8.

Table 2
Influence of different pH gradients on R and F_{eff}

Parameter	Protein	pH gradient			
		3–10	4–7	4–5	5–8
F_{eff}	Ovalbumin	12.3	12.7	19.0	6.1
	Bovine serum albumin (BSA)	19.0	21.3	21.3	17.7
	Haemoglobin	11.0	3.0	1.13	4.24
R	Ovalbumin–BSA	0.66	0.83	1.13	0.82
	Ovalbumin–haemoglobin	3.2	3.46	3.11	2.97
	BSA–haemoglobin	2.89	2.48	1.38	2.24

The values were calculated from IEF of a mixture containing ovalbumin, bovine serum albumin (BSA) and haemoglobin as described in Fig. 10.

proteins (Fig. 10) were undertaken. To estimate F_{eff} values, each protein was individually focused in a separate experiment. The calculated constants F_{eff} and R inferred from Fig. 10 are summarized in Table 2. Compared with bovine serum albumin and ovalbumin, F_{eff} for haemoglobin is smaller in each of the Servalyte types used. This may be caused by the significantly smaller value of the mobility slope ($d\mu/dpH$) for haemoglobin (Table 1). According to Eq. 6, the influence of different pH gradients on F_{eff} is negligible if the pH gradients show a linear behaviour, as the pH gradients for albumin did (Fig. 10C).

On the other hand, non-linear pH gradients deteriorate F_{eff}, as is found for haemoglobin within a pH gradient between 4–7 and 4–5 or ovalbumin within a pH gradient between 5 and 8 pH units. As expected from Eq. 10, for the separation of bovine serum albumin and ovalbumin an improvement in resolution with decreasing pH gradient is fulfilled in practice.

3.8. Influence of different ampholytes on resolution

It is possible that the effects produced by additives may differ based on the chemistry of the ampholytes used. Therefore, we investigated the influence of different ampholytes [Servalyte 5–8, Bio-Lyte 5–8 and Ampholine 5–8, 0.35% (v/v) solutions] on R in the free-flow IEF of the protein standard mixture with and without addition of 3% (w/v) of saccharose. The ampholytes used produced minor differences in the declared pH gradients. The resolution increased in each instance on addition of 3% of saccharose (Table 3).

Table 3
Influence of different ampholytes on resolution

Conditions	Resolution		
	Bio-Lyte	Pharmalyte	Servalyte
Without additives	1.35	1.42	1.67
With 3% saccharose	2.3	1.93	2.11

The values were calculated from IEF of a mixture containing bovine serum albumin (BSA) and human haemoglobin. Experimental conditions: temperature of chamber, 5°C; medium, 0.35% (v/v) ampholyte with and without 3% (w/v) saccharose; sample solution, 1 mg ml^{-1} BSA and haemoglobin in medium; electric field strength, 175 V cm^{-1}; transit time, 7 min.

4. Conclusions

The separation of a protein mixture consisting of bovine serum albumin, ovalbumin and haemoglobin by preparative continuous free-flow IEF was mainly characterized by two parameters: resolution and focusing efficiency. Hydrophilic additives to the IEF medium can effectively control the resolution and focusing efficiency of proteins in free-flow IEF and caused up to an

eightfold improvement in resolution. This effect is mainly caused by the influence of these additives on electrodispersive effects. Shallow pH gradients have a minor influence on the focusing efficiency, but improve the resolution. For the proteins investigated, transit times as short as ca. 7 min gave optimum resolution. These limited transit times may be very helpful for separating biomolecules having a short lifetime.

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