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Use of discrete spacers for the separation of proteins by gel isotachophoresis

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ABSTRACT

Discrete amphoteric spacers can generate a linear pH gradient concomitant with a conductivity gradient on isotachophoresis. The design of the system requires a mixture of discrete spacers calculated to generate an electrophoretic mobility–pH or charge–pH relationship as linear as possible and a mixture of counter ions calculated to provide a buffer capacity proportional to the charge of the mixture of spacers over the expected pH gradient. The separation of human blood plasma proteins in agarose gels is used as an example. The spacers were amphoteric ions: amino acids, amino acid derivatives and sulphonic compounds. The results obtained using mixtures of discrete spacers resemble those obtained using commercially available ampholyte mixture as judged from the separation of the C3-complement proteins. The major advantage of the use of discrete spacers in ITP lies in the possibility of designing separation systems for specific proteins.

INTRODUCTION

Commercially available ampholyte mixtures have been successfully used for the separation of proteins by isotachophoresis (ITP) in agarose gels [1]. However, these mixtures are not defined in terms of the individual components. The object of this study was to investigate the requirements of the ITP system in order to obtain optimum protein separations in agarose gels by the use of discrete spacers.

The principle used for the design of isotachophoretic systems for the separation of proteins using commercially available ampholyte mixtures is that a pH gradient concomitant with a conductivity gradient should be generated. The approach is based on the following considerations: (1) On isoelectric focusing (IEF), proteins are separated in a pH gradient, generated by ampholytes and stabilized between the anode and the cathode; on ITP, proteins are separated in an electrophoretic mobility or conductivity gradient, generated by spacers and stabilized between the leading ion and the terminating ion; (2) graphical representation of the conductivity *versus* the distance in IEF will show small rises and falls in conductivity centred around a horizontal line in a wavy pattern. The hills of increased conductivity originate from the charges of the different ampholytes used to generate the pH gradient. The corresponding graph for ITP would then be like a staircase starting from the low conductivity of the terminating ion and rising to the high conductivity of the leading ion. The

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individual steps would represent the conductivity of the different spacer ions. The difference between these two curves is the increase in charge of the ampholytes in the latter. Therefore, to obtain a pH gradient concomitant with a conductivity gradient, one should have counter ions that can supply the charge to the ampholytes in a pH gradient [1]. In this way, proteins can be focused by ITP not only at their isoelectric points but also at almost any point on their titration curves. The advantage of this lies in the fact that the solubility of most proteins is lowest at their isoelectric points and some native proteins are not stable. This has been illustrated for plasma proteins [2] and particularly for C3-complement proteins in a recent study [3]. In ITP the slope of the pH gradient generated can also be regulated to increase the resolution as in IEF.

EXPERIMENTAL

Human plasma samples were supplied by the blood centre at the Karolinska Hospital. Agarose, electrode strips and paper sample applicators were obtained from Pharmacia (Uppsala, Sweden), nitrocellulose membrane Immobilon from Millipore (Bedford, MA, U.S.A.), rabbit immunoglobulins (Igs) and horsedish peroxidase-conjugated swine antibodies against rabbit Igs from Dakopatts (Copenhagen, Denmark), Gelbond from FMC Bioproducts (Rockland, ME, U.S.A.), swine serum from Flow Labs. (Uxbridge, Middlesex, U.K.) and dry defatted milk from Semper (Stockholm, Sweden). All chemicals were of analytical-reagent grade.

Isotachophoretic system

The procedure was basically as described previously [1]. Isotachophoresis was run in flat-bed gels. The plasma proteins were electrophoresed towards the anode in 1% agarose IEF, 12% sorbitol gels, on a flat-bed electrophoresis apparatus (Pharmacia FBE3000). The electrode solutions were absorbed in the electrode strips, placed on the edges of the gel. The gels were prerun at 5–10 W until the isotachophoretic front reached 1.0–1.5 cm from the cathode. Then the samples, 20 μl of 20% plasma in 100 m*M* Tris–HCl (pH 8.0), were applied on paper sample applicators, placed behind the front. Electrophoresis was carried out at 5–20 W (300–1200 V) until the front reached the anode. The leading ion, the counter-ions and the mixture of spacers were included in the gel mixture. The terminating ion (10 ml) was included in the cathode. The anode solution (20 ml) contained the counter-ions ten times more concentrated than in the gel and adjusted to pH 6 with sulphuric acid. The gels were stained with Coomassie Brilliant Blue or immunostained. In the latter instance, the proteins from the agarose gel were transferred to a nitrocellulose filter (NCF) by overlaying the gel with a stack consisting of a wet NCF, a sheet of wet blotting paper, eight layers of dry blotting paper (Munktell, Stora, Sweden), a glass plate and, on the top, a 5-kg weight, for 20 min at room temperature. The immunological detection of proteins on NCF was performed essentially according to Towbin *et al.* [4], except that 5% dry defatted milk in 0.9% NaCl–10 m*M* Tris–HCl buffer (pH 7.4) (TBS) was used instead of bovine serum albumin. The Igs were diluted 1:1000 in 5% dry defatted milk–10% swine serum in TBS. The peroxidase-labelled antibodies were stained with 4-chloro-1-naphtol [5].

Spacers

The spacers were amphoteric ions such as amino acids, amino acid derivatives and sulphonic compounds as listed in Table I. The pK_a and pK_b values were determined by titration at 20°C. The criteria for the selection of the amphoteric ions were based on the pK_b value of the basic group and the commcercial availability. The concentration of the spacers in the mixture was calculated to generate a charge–pH or a mobility–pH relationship as linear as possible (a constant slope) in the pH interval between the two extreme pK_b values. For the mobility values the factor charge × (molecular weight)$^{-\frac{1}{2}}$ was used in the calculations, according to the approximation of Jokl [6].

TABLE I

CHARACTERISTICS OF SPACERS

Ion[a]	M.W.	$pK_{a,20°C}$	$pK_{b,20°C}$	Mixture[b]	Mixture[c]
MES[d]	195.2	1.3	6.1	23.21	21.81
ACES[d]	182.2	1.3	6.75	0.83	5.8
BES[d]	213.3	1.35	7.1	3.36	3.62
MOPS[d]	209.3	1.4	7.2	4.19	3.1
TES[d]	229.3	1.3	7.45	2.99	2.2
DIPSO[d]	243	1.3	7.5	2.61	2.07
HEPES[d]	238.3	3.05	7.5	2.61	2.07
TAPSO[d]	259.3	1.3	7.58	1.96	1.93
HEPPSO[d]	268.3	3.5	7.9	0.92	1.87
EPPS[d]	252.3	3.75	7.9	0.92	1.87
POPSO[d]	362.4	3.9	7.9	0.92	1.87
Tricine	179.2	2.15	8.05	1.07	2.1
GLYGLY	132.1	3.15	8.2	1.58	2.59
Bicine	163.2	1.95	8.25	1.97	2.74
TAPS[d]	243.3	1.3	8.4	3.4	3.44
Asparagine	150.1	2.25	8.85	7.95	4.4
Serine	105.1	2.21	9.15	4.03	3.99
Glutamine	146.2	2.35	9.15	4.03	3.99
Glycine	75.05	2.34	9.6	2.84	3.62
Alanine	89.1	2.35	9.87	5.55	3.06
β-Alanine	89.1	3.55	10.29	6.2	2.07
GABA	103.1	4.03	10.56	4.48	3.44
EACA	131.2	4.37	10.8	12.39	16.37

[a] Abbreviations: MES = 2-(N-morpholino)ethanesulphonic acid; ACES = N-(2-acetamido)-2-aminoethanesulphonic acid; BES = N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid; MOPS = 3-(N-morpholino)propanesulphonic acid; TES = N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; DIPSO = 3-[N,N-bis(2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; TAPSO = 3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulphonic acid; HEPPSO = N-(2-hydroxyethyl)piperazine-N′-(2-hydroxypropanesulphonic acid); EPPS = N-(2-hydroxyethyl)piperazine-N′-(3-propanesulphonic acid); POPSO = piperazine-N,N′-bis(2-hydroxypropanesulphonic acid); GLYGLY = glycylglycine; TAPS = N-tris(hydroxymethyl)methyl-3-aminopropanesulphonic acid; GABA = γ-aminobutyric acid; EACA = ε-aminocaproic acid.

[b] Mixture 1 in mol%, calculated to generate a linear charge–pH relationship.

[c] Mixture 2 in mol%, calculated to generate a linear mobility–pH relationship [6].

[d] Good buffers [7].

Calculations

The proportions of the ions in the mixture were calculated by an iterative procedure with an accuracy of 0.01 pH unit over the pH interval of interest. The optimum result would be a constant slope for the charge–pH or mobility–pH graph. At the start of the calculations equal concentrations were allotted to all components in the mixture. In order to adjust the graph to a first-order linear relationship between charge and pH, the concentration of each particular ion was varied. The given value was multiplied by a factor proportional to the difference between the value for the slope of the calculated graph at the pK_b of the ion and the median of the slope for the overall graph. The process was repeated several times, until the individual additons to the generated slope for each spacer did not vary by more than 1%. The compositions of the mixtures of spacers obtained after such calculations are given in Table I (last two columns).

A mixture of bases as counter ions was chosen and calculated in a similar manner to the spacer ions. The concentration of the leading ion (glutamic acid) was just above that of the first spacer used and the total concentration of counter ions was about 50% of the total concentration of the leading ion and the spacers in order to obtain a pH value near the pK_b of the spacers. The terminating ion, absorbed in the cathode, was 200 m*M* lysine.

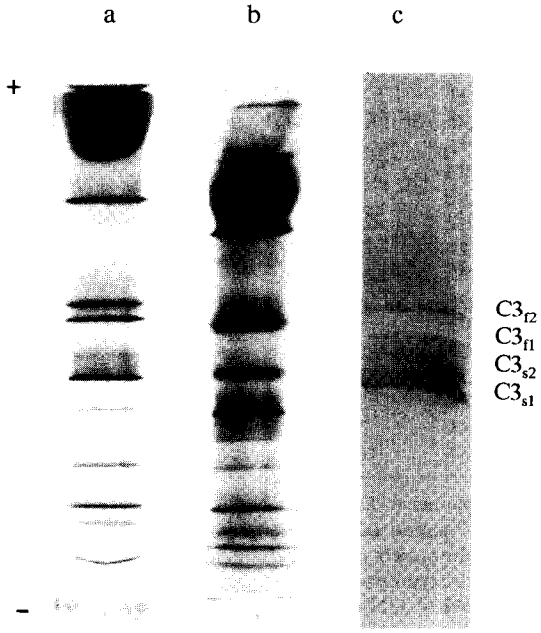

Fig. 1. Agarose gel ITP of human blood plasma proteins. The size of the gels was 12 × 12 × 0.1 cm. (a) The mixture of spacers was calculated to generate a linear charge–pH relationship (Table I, column 5, mixture 1). Leading ion, 40 m*M* glutamic acid; terminating ion, lysine; spacers, 250 m*M* of mixture 1; counter ions, 100 m*M* bistris, 34 m*M* tris and 17 m*M* 2-aminoethanol. Coomassie Brilliant Blue staining. (b) The mixture of spacers was calculated to generate a linear mobility–pH relationship (Table I, column 6, Mixture 2). All other ions as in (a). (c) Immunoblot stained with antibodies against $C3_c$-complement factor after ITP of human blood plasma in a gel similar to that illustrated in (a).

Pieces of 0.5 cm (along the electrophoresis path) × 2 cm (wide) were cut and left overnight in 1 ml of water for pH and conductivity measurements.

RESULTS

Two different calculations for the composition of the mixture of spacers were made, one to obtain a linear charge–pH relationship and the other to obtain a linear mobility–pH relationship. Fig. 1 illustrates the results after agarose gel isotachophoresis of human blood plasma proteins using mixtures of discrete spacers calculated as described above. No major difference was observed between the two gels. Lane c shows the result after immunodetection of C3 components in a gel identical with that illustrated in lane a. Four different bands were stained, in agreement with earlier reported results using ampholyte mixtures as spacers [3].

The conductivity and pH measurements in gels identical with those illustrated in Fig. 1, except for a longer electrophoretic path, are shown in Fig. 2. An almost linear pH gradient concomitant with a conductivity gradient was obtained in both instances.

DISCUSSION

The best results for the separation of plasma proteins by ITP using discrete spacers were obtained when the mixture of spacers was such that it could generate a pH–charge relationship as linear as possible and the counter ions were such that they could provide a buffer capacity corresponding to the charge of the mixture of spacers over the expected pH interval.

The pH gradient generated in ITP by using mixtures of spacers calculated upon mobility did not differ from the gradient generated by using mixtures of spacers calculated upon charge (Fig. 2). One reason for this is the small differences in molec-

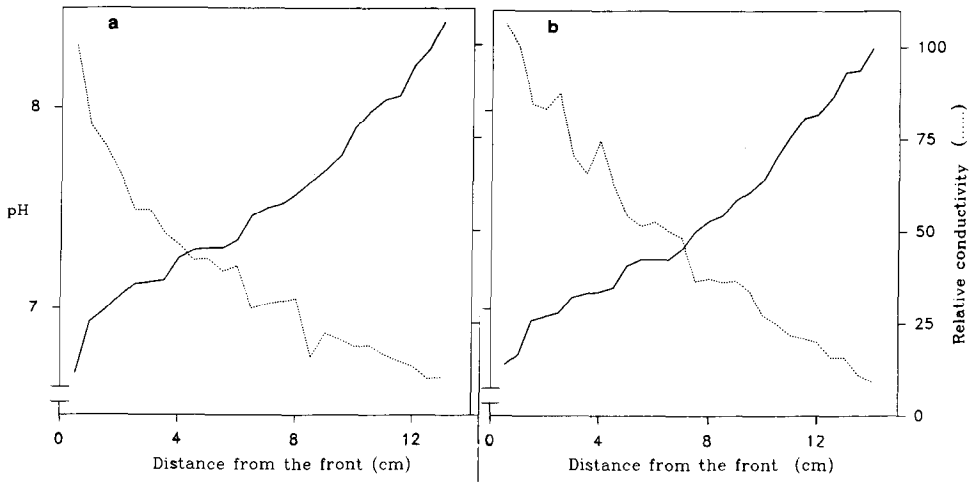

Fig. 2. pH and relative conductivity for gels identical with those illustrated in Fig. 1a and b, respectively, except that the size of the gels was 24 × 12 × 0.1 cm and ITP was run along the length.

ular weight among the spacers used. However, the conductivity gradient generated in ITP in the former instance was close to a straight line.

The short electrophoretic path required to obtain a pH gradient concomitant with a conductivity gradient is one of the major advantages of the present system. This is particularly useful for preparative purposes where the size of the equipment is usually a limiting factor. In a single lane (Fig. 1), as much as 10 μl of plasma per millilitre of gel were separated. In addition, isotachophoretic systems with narrower pH intervals, designed for the separation of specific proteins, or using a particular set of discrete spacers, may be calculated using the general principles described here.

CONCLUSIONS

The selection of appropriate mixtures of discrete spacers and counter ions determines the quality of the separation of proteins by gel ITP. The main requirements for the use of discrete spacers in isotachophoresis for the separation of proteins are a pH–charge relationship as linear as possible for the mixtures of spacers, the counter ions should provide a buffer capacity paralleling the charge of the mixture of spacers and the proportions between the spacers and the counter ions should be close to 2.

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