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Journal of Chromatography A, 719 (1996) 159–170

JOURNAL OF CHROMATOGRAPHY A

Effect of polyamines on the separation of ovalbumin glycoforms by capillary electrophoresis

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Abstract

The successful separation of ovalbumin (M_r 45 000; p*I* 4.7) glycoforms by capillary electrophoresis in an uncoated fused-silica capillary with different buffer additives is reported. The optimum conditions for obtaining the resolution of glycoforms were 25 m*M* borate (pH 9.0) containing 0.87 m*M* spermidine or 0.14 m*M* spermine. The effects of different concentrations of putrescine, cadaverine, spermidine, spermine and some monoamines or diamines are compared in terms of selectivity factors of ovalbumin peaks. Addition of sodium dodecyl sulfate at a concentration below the critical micelle concentration increased the resolution between the three main peaks of ovalbumin but did not permit their microheterogeneity to be expressed.

1. Introduction

Capillary electrophoresis (CE) has developed rapidly since Jorgenson and Lukacs' pivotal work [1] in the early 1980s and has been widely applied in the fields of biochemistry and molecular biology. Molecules such as proteins and oligonucleotides have been separated under conditions that provide numbers of theoretical plates in the hundreds of thousands and more.

Practitioners of CE have realized that proteins present unique challenges to the separation method, owing to their inherent tendency to adsorb to the inner walls of fused-silica capillaries. Such adsorption leads to considerable peak broadening and asymmetry, making it difficult to attain the impressive efficiencies predicted by theory. Several efforts have therefore been made to prevent adsorption and improve protein separation capabilities. The strategies undertaken can be grouped into two main categories: (i) chemically bonding a neutral material to the inner walls of fused-silica capillaries and (ii) manipulation of buffer pH and ionic strength to cause the proteins and capillary walls to experience coulombic repulsion. In the category of buffer manipulation, low-pH phosphate buffers were used by McCormick [2] for the purpose of reducing the negative charge of fused silica and for inducing some protective screening of the silica surface by phosphate groups. Lauer and McManigill [3] employed high-pH buffers with added ionic modifiers to create negatively charged proteins which will then be repelled by the negatively charged capillary walls. In the case of capillary coating materials, their long-term stability under alkaline conditions appears to be poor [2,4]. Much of the work has used the open-tubular mode referred to as free solution capillary electrophoresis or capillary zone electrophoresis (CZE), since it exhibits

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0021-9673/96/$15.00
SSDI 0021-9673(95)00337-1

simplicity, high separation power and the ability to perform rapid and automated analysis.

Ovalbumin is a glycoprotein of avian egg white. It is 385 amino acids in length and has a molecular mass (M_r) of about 45 000 and two possible asparagine-linked glycosylation sites [5]. As with the carbohydrate component of most glycoproteins, the ovalbumin carbohydrate structure is very heterogeneous [6]. Nine different oligosaccharide chains have been identified [7,8], all of which have been classified as high-mannose or hybrid structures. The hybrid and high-mannose forms are present in a ca. 1:1 ratio.

The microheterogeneity of glycoproteins has been examined by methods of electrofocusing and isoelectric focusing, but they have limitations. An alternative to these traditional methods of separation is the use of high-performance capillary electrophoresis (HPCE), a technique that combines reproducibility and high efficiency. Landers et al. [9] demonstrated the utility of HPCE for the analysis of ovalbumin microheterogeneity by using putrescine as a modifier of endo-osmotic flow (EOF). Recently, Oda et al. [10] described the effectiveness of alkyl compounds with quaternary ammonium moieties in resolving glycoprotein isoforms. Seven to eight isoforms of chorionic gonadotropin were separated with baseline resolution in the presence of 1,3-diaminopropane as a buffer additive [11].

Surfactants are among the most widely used buffer additives in HPCE. Numerous types of surfactants, that is, anionic, cationic, zwitterionic or non-ionic, have been used. Sodium dodecyl sulfate (SDS), an anionic surfactant, may interact with proteins in limited amounts. SDS may bind and ion pair, bind and modify the net charge, or bind and modify the tertiary structure or activity. Plasma apolipoproteins (HDL, LDL) were characterized by Tadey and Purdy [12] using borate buffer to which 0.1% SDS was added.

In this paper, we describe the utility of CZE for the determination of ovalbumin microheterogeneity when polyamines (PAs), such as putrescine (PUT), spermidine (SPD), spermine (SPM) and cadaverine (CAD) are employed as buffer additives. We also compared the effect of PAs with that of a diamine, agmatine (AGM), or monoamines, such as hydroxylamine (HAM) or ethylamine (ETAM), in resolving microheterogeneity. The effect of SDS in the running buffer, at a concentration below the critical micelle concentration (CMC), was also studied. It is demonstrated that changes in selectivity are the consequence of many factors, including M_r, concentration, molecular structure, etc., of the modifier and not of the EOF itself.

2. Experimental

2.1. Chemicals

All chemicals used for the preparation of the buffer, boric acid and borax (sodium tetraborate) were of analytical-reagent grade (Merck, Darmstadt, Germany) and were used as received. SDS was obtained from ICN Biochemicals (Montreal, Canada). Deionized water was doubly distilled and filtered through Millipore (Bedford, MA, USA) GS filters of 0.22 μm pore diameter (DIBDF-water). HAM, ETAM, AGM, PUT, SPD, SPM and CAD were obtained from Sigma (St. Louis, MO, USA). Ovalbumin (M_r 45 000) and bovine serum albumin (BSA) (M_r 47 000) were purchased from Serva (Heidelberg, Germany), kit No. MS II for non-denaturing systems. The neutral marker, benzene, was obtained from Merck.

2.2. Instrumentation

CZE was performed using a Spectraphoresis 500 system from Thermo Separation Products (Fremont, CA, USA). Microbore fused-silica tubing, coated with polyimide (Scientific Glass Engineering, Milton Keynes, UK) of 75 μm I.D. and 190 μm O.D., with a total length of 69 cm and a separation length of 63 cm, was used. The capillary was enclosed in a casette for easy handling. On-line detection was performed with a variable-wavelength UV–Vis detector of bandwidth 6 nm (Thermo Separation Products). Detection of proteins was monitored at 200 nm and electropherograms were recorded using a SP 4290 integrator (Thermo Separation Products).

2.3. Capillary electrophoresis separation conditions

A new capillary was first conditioned with 0.5 *M* NaOH for 5 min at 60°C, 0.05 *M* NaOH for 5 min at 60°C and DIBDF-water for 5 min at 60°C. Equilibration of the capillary was then performed by washing it with 2.5, 25 or 100 m*M* sodium borate buffer (pH 9.0) for 15 min at 25°C. After this, the capillary was washed again with the same buffers for 15 min at 25°C under an applied voltage of 11 kV. Finally, the capillary was washed with the corresponding concentration of amine, PA and/or SDS for 90 min at 25°C. Regeneration of the capillary surface between runs was performed by rinsing it in the following sequence: 0.05 *M* NaOH for 3 min, DIBDF-water for 5 min and 2.5, 25 or 100 m*M* sodium borate buffer (pH 9.0) for 3 min.

Protein solutions of 0.95 μM ovoalbumin (p*I* = 4.7) or 0.64 μM BSA (p*I* = 4.9), prepared in the electrophoresis buffer diluted threefold, were injected into the capillary by siphoning for a fixed time of 9 s. Benzene at a concentration of 4% (v/v) in the same diluted buffer was used as a neutral marker. All electrophoretic separations were carried out at a constant voltage of 11 kV and the capillary temperature was maintained at 25 ± 0.01°C by using an oven Peltier high-speed fan and resistive thermal device.

2.4. Data analysis

Samples of ovalbumin and BSA were analysed at least four times under each set of analytical conditions and the average time for each peak was determined. Data were analysed using a BMDP-2V ANOVA program and the mean values were compared using Duncan's multiple range test.

3. Results and discussion

3.1. HPCE of ovalbumin in the presence or absence of monoamines

HPCE analysis of ovalbumin was carried out using a 69 cm × 75 μm I.D. capillary and 25 m*M* sodium borate (pH 9.0) as running buffer. This buffer system was chosen in order to produce a pH value higher than the isoelectric point (p*I*) of ovalbumin to be separated. This renders the protein negatively charged, resulting in repulsion from the charged fused-silica capillary walls and thereby minimizing adsorption [13]. The borate ion may also be inherently better for analysing glycoproteins [9] because of its potential to form a borate–sugar diol complex, as proposed by Novotny and co-workers [14,15].

The electrophoretic profile of ovalbumin is shown in Fig. 1A. The main peak at 16.4 min (No. 6) was flanked on the left side by another peak at 15.7 min (No. 1) with slightly different charge-to-mass ratio. The selectivity factor between these two peaks ($\alpha_{6,1}$) was 1.04 (Table 1). Addition of 2.11 m*M* HAM to the running buffer had no effect on either EOF or selectivity, although an increase in its efficiency was observed (Fig. 1B and Table 1). The profile in Fig.

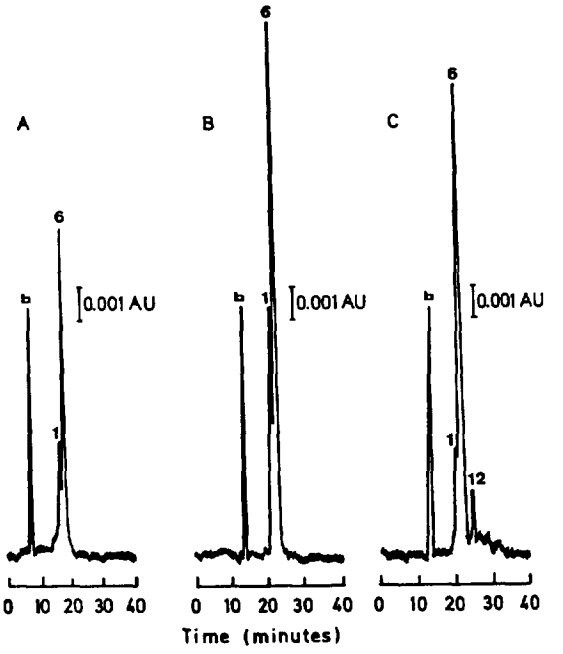

Fig. 1. HPCE separation of ovalbumin. Electrophoresis was carried out in a 69 cm × 75 μm I.D. (63 cm to the detector) capillary in (A) the absence or the presence of (B) 2.11 m*M* HAM or (C) 34.5 m*M* ETAM in 25 m*M* borate buffer (pH 9.0). Separation conditions: 11 kV, 25°C. b = Benzene used as neutral marker.

Table 1
Effect of amines, polyamines and SDS, as modifier, on EOF and resolution of ovalbumin in a 69 cm × 75 μm I.D. uncoated capillary

Concentration of running buffer[a] (m*M*)	Modifier	Concentration of modifier (m*M*)	Current (μA)	Migration time of neutral marker, t_0 (min)	Migration time of ovalbumin peaks (min)			Selectivity factor	
					1[c]	6	12	$\alpha_{6,1}$[c]	$\alpha_{12,6}$[c]
25	–	–	30	12.3 ± 0.2[b]	15.7 ± 0.2[b]	16.4 ± 0.2[b]	–	1.04	–
25	HAM	0.10	31	11.5 ± 0.3	15.6 ± 0.2	16.2 ± 0.3	–	1.05	–
25	HAM	2.11	31	13.1 ± 0.2	20.1 ± 0.3	21.2 ± 0.4	–	1.05	–
25	ETAM	34.50	45	13.3 ± 0.4	19.5 ± 0.5	20.6 ± 0.7	22.5 ± 0.4[b]	1.06	1.09
25	ETAM	200.00	58	23.1 ± 0.5	27.0 ± 0.4	27.4 ± 0.4	29.6 ± 0.5	1.01	1.08
25	PUT	1.83	29	17.6 ± 0.6	33.3 ± 0.5	36.3 ± 0.5	–	1.09	–
25	PUT	5.48	31	24.4 ± 0.5	55.3 ± 0.5	64.1 ± 0.6	73.5 ± 0.6	1.16	1.15
25	CAD	5.48	32	23.9 ± 0.3	51.8 ± 0.3	60.4 ± 0.4	69.1 ± 0.5	1.17	1.14
25	CAD	7.00	35	26.9 ± 0.3	57.5 ± 0.3	69.4 ± 0.3	155.9 ± 0.7	1.21	2.25
25	AGM	0.14	27	13.1 ± 0.6	18.7 ± 0.5	19.5 ± 0.5	–	1.04	–
25	AGM	5.48	31	25.7 ± 0.4	55.0 ± 0.3	60.7 ± 0.4	63.8 ± 0.4	1.10	1.05
25	SPM	0.07	29	29.7 ± 0.5	68.9 ± 0.3	101.6 ± 0.4	119.6 ± 0.4	1.47	1.18
25	SPM	0.14	30	31.5 ± 0.5	67.6 ± 0.5	116.9 ± 0.5	143.8 ± 0.6	1.72	1.23
25	SDS	0.034	28	12.1 ± 0.2	17.7 ± 0.2	18.7 ± 0.2	–	1.05	–
25	SDS	0.34	27	12.1 ± 0.1	16.8 ± 0.1	18.3 ± 0.1	20.8 ± 0.2	1.09	1.14
25	SDS	7.00	28	12.1 ± 0.2	28.8 ± 0.2	31.1 ± 0.2	38.3 ± 0.2	1.08	1.23
2.5	SDS	0.034	15	10.6 ± 0.2	15.2 ± 0.1	15.8 ± 0.1	–	1.04	–
100	SDS	0.34	89	17.0 ± 0.3	25.9 ± 0.2	30.2 ± 0.3	37.9 ± 0.3	1.17	1.25

[a] Sodium borate (pH 9.0).
[b] Standard deviation ($n = 5$).
[c] Peak number in Figs. 1, 3, 6 and 7.

1C represents the electrophoretic separation of the same ovalbumin solution when 34.5 m*M* ETAM was added to the running buffer. There was very little effect on EOF, as determined from the migration of benzene at 13.3 min, by this addition, although the current through the capillary increased to 45 μA. Another peak could be detected at 22.5 min (No. 12). The selectivity ($\alpha_{12,6}$) was 1.09. Higher concentrations of ETAM, up to 200 m*M*, only reduced the EOF but did not affect either the selectivity factors or the resolution of ovalbumin microheterogeneity. Fig. 2 illustrates the structure of monoamines, diamines and PAs used as buffer additives throughout this work. Both HAM and ETAM has a positive charge at the N-terminus of each molecule, able to cover free silanol groups on the inner wall of the capillary, but, whereas HAM exposes a negative charge to the exterior, ETAM has no charge at its end. Hence the slight ionic interaction between HAM and protein increases the efficiency of the ovalbumin

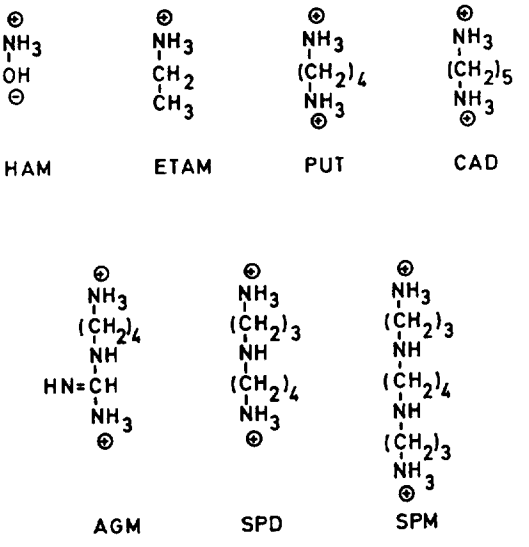

Fig. 2. Structures of hydroxylamine (HAM), ethylamine (ETAM), putrescine (PUT), cadaverine (CAD), agmatine (AGM), spermidine (SPD) and spermine (SPM) used as buffer additives.

peaks whereas the hydrophobic interaction between the short alkyl chain of ETAM and the hydrophobic moieties of the protein increases its retention time.

3.2. HPCE of ovalbumin in the presence of diamines and polyamines

Electrophoretic separations of an ovalbumin sample with 25 m*M* borate buffer containing AGM, CAD, PUT or SPM are shown in Fig. 3A–D. Addition of 5.48 m*M* AGM to the running buffer results in the resolution of six components present in the sample (Fig. 3A). A lower concentration of AGM, 0.14 m*M*, did not produce the same effect (Table 1). The imino group of AGM, near the terminal, exterior $-NH_3^+$ group, possesses an accumulation of negative charge by induction, since the electronegativity of nitrogen is 2.3 compared with 2.1 for the carbon atom. This implies that the net positive charge of the neighbouring amino group was partially neutralized and thus the electrostatic interaction with an anionic protein diminishes. The resolution of a multitude of components, up to thirteen, present in ovalbumin was achieved when 5.48 m*M* CAD (Fig. 3B), 5.48 m*M* PUT (Fig. 3C) or 0.14 m*M* SPM (Fig. 3D) were used as additives. Concomitant with the enhanced resolution was a decrease in EOF, calculated by the migration time of benzene, ranging from 25 to 30 min (Table 1). The selectivity factors $\alpha_{6,1}$ and $\alpha_{12,6}$ increased with respect to that obtained in the absence of modifiers. The optimum conditions for separation resulted in the choice of 25 m*M* borate buffer with 0.14 m*M* SPM as the running buffer. The current generated with this buffer was 30 μA, similar to the other conditions of analysis (Table 1).

It has been hypothesized that diaminoalkanes such as AGM, PUT, SPD and SPM enhance the separation of glycoforms by decreasing the EOF [3,9]. This decrease in EOF results from the cationic amines (Fig. 2) interacting with the negatively charged free silanol groups on the capillary wall and thereby increasing the migration time of the sample. This increase in migration time allows an enhanced resolution of the different glycoforms. PUT has also been used as a buffer additive for the determination of the microheterogeneity of glycoproteins, including tissue plasminogen activator [16], ovalbumin and pepsin [9] and human recombinant erytropoietin [17]. Oda et al. [10] demonstrated that α,ω-bisquaternary ammonium alkanes allowed the determination of microheterogeneity and were more efficient than their diamino counterparts.

It seems that chemical properties, bifunctional character and length of the alkyl chainall, play a role in the effectiveness of glycoform resolution. It was therefore of interest that AGM, with an alkyl chain similar to CAD, does not produce the same effect since not all the ovalbumin glycoforms were resolved. This has been explained here on the basis of the imino group of AGM, which is not present in other amines. Hence the hydrophilic interaction between AGM and protein was reduced and the EOF increased.

3.3. Effect of SPD concentration on the EOF and resolution of ovalbumin microheterogeneity

The addition of increasing SPD concentrations, from 0.087 to 0.87 m*M*, to the running buffer had the expected effect, that is, a significant slowing of the EOF and a subsequent increase in the resolution of several peaks obtained from the ovalbumin preparation (Table 2 and Fig. 4). Whereas the addition of 0.087 m*M* SPD produced only an increase in the selectivity factor, $\alpha_{6,1} = 1.08$, with respect to that obtained in its absence, $\alpha_{6,1} = 1.04$ (Figs. 1A and 4A), an SPD concentration two times higher, 0.17 m*M*, promoted an increase in both the selectivity factor, $\alpha_{6,1} = 1.13$, and the resolution of several peaks of ovoalbumin (Fig. 4B and Table 2). On increasing the concentration of SPD in the running buffer to 0.87 m*M*, the resolution of the protein component was optimum. The dramatic increase in resolution resulted from the decrease in EOF, indicated by a migration time of the neutral marker of 26.0 min (Table 2). As many as thirteen peaks are reproducibly observed between 60 and 90 min. Very similar results were obtained by Landers et al. [9] using PUT as an

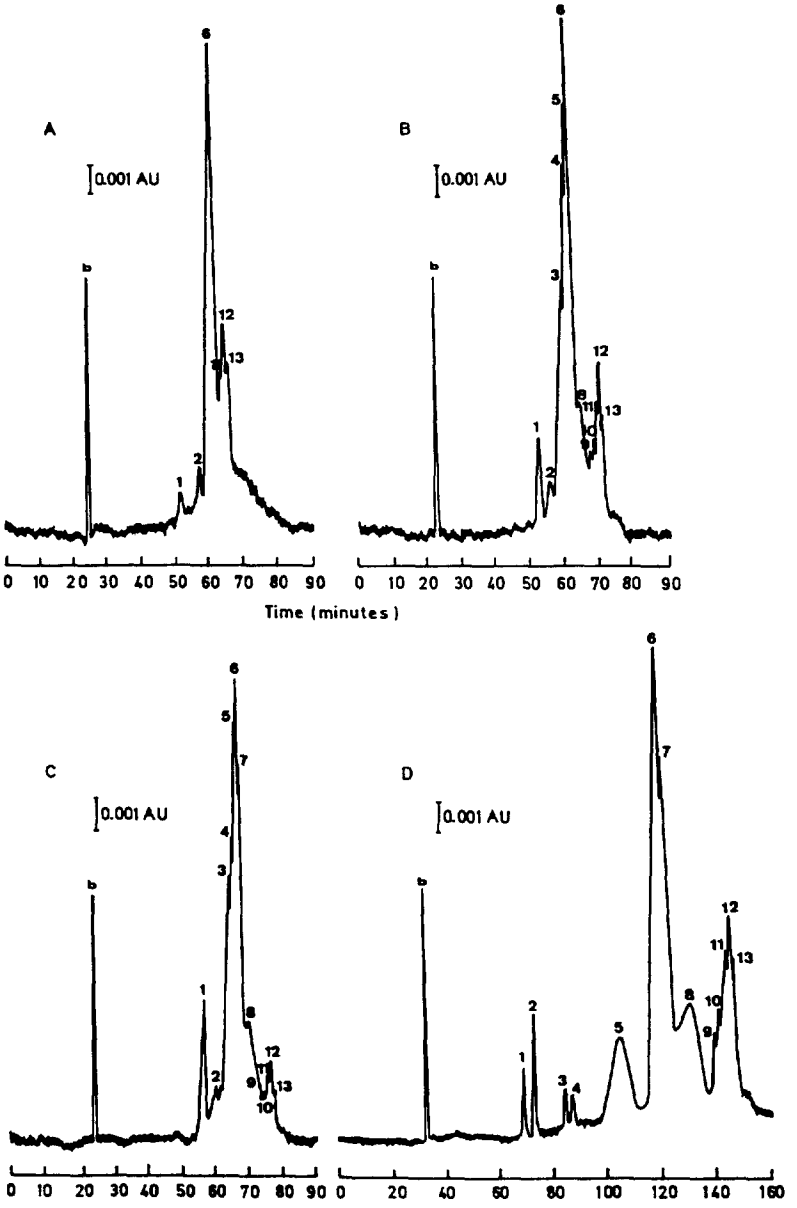

Fig. 3. HPCE separation of ovalbumin. Electrophoresis was carried out in a 69 cm × 75 μm I.D. (63 cm to the detector) capillary in the presence of (A) 5.48 m*M* AGM, (B) 5.48 m*M* CAD, (C) 5.48 m*M* PUT or (D) 0.14 m*M* SPM in 25 m*M* borate buffer (pH 9.0). Separation conditions: 11 kV, 25°C. b = Benzene used as neutral marker.

additive. Nevertheless, they used longer capillaries (87 cm) of 50 μm I.D., and obtained shorter migration times, about 45 min, for ovalbumin glycoforms.

To rule out the possibility that the increase in SPD concentration added to the running buffer has a non-specific effect on ovalbumin, the separation of a non-glycosylated protein having roughly the same size and p*I* was carried out. BSA, a non-glycosylated protein of $M_r = 47\,000$,

Table 2
Effect of SPD concentration on EOF and resolution of ovalbumin in a 69 cm × 75 μm I.D. uncoated capillary

Concentration of running buffer[a] (m*M*)	Concentration of SPD (m*M*)	Current (μA)	Migration time of neutral marker, t_0 (min)	Migration time of ovalbumin peaks (min)			Selectivity factor	
				1[c]	6	12	$\alpha_{6,1}$[c]	$\alpha_{12,6}$[c]
25	–	30	12.3 ± 0.1[b]	15.7 ± 0.2[b]	16.4 ± 0.3[b]	–	1.04	–
25	0.087	27	13.2 ± 0.1	31.9 ± 0.1	31.9 ± 0.2	–	1.08	–
25	0.170	27	20.6 ± 0.2	41.2 ± 0.2	46.7 ± 0.2	–	1.13	–
25	0.520	29	24.1 ± 0.4	48.5 ± 0.3	57.4 ± 0.3	61.8 ± 0.3[b]	1.18	1.07
25	0.700	29	25.9 ± 0.4	56.9 ± 0.5	67.1 ± 0.4	79.3 ± 0.5	1.18	1.18
25	0.870	29	26.9 ± 0.6	58.1 ± 0.5	68.6 ± 0.6	85.5 ± 0.5	1.18	1.24
100	–	89	17.0 ± 0.1	27.2 ± 0.1	28.5 ± 0.2	–	1.04	–
100	0.870	85	27.0 ± 0.1	57.4 ± 0.1	64.3 ± 0.2	73.6 ± 0.3	1.12	1.14

[a] Sodium borate (pH 9.0).
[b] Standard deviation ($n = 5$)
[c] Peak number in Figs. 4 and 7.

having a p*I* of 4.9, was separated in 25 m*M* borate containing 0.087–0.87 m*M* SPD (pH 9.0). It migrated as a single, intact peak with no indication of resolving a multitude of peaks observed for ovoalbumin (Fig. 5). The migration time of the BSA peak increased from 22 min (Fig. 5A) to 52 min (Fig. 5C), with very low efficiency.

3.4. HPCE of ovalbumin in the presence of SDS, an anionic surfactant

It was of interest to determine whether an anionic surfactant containing protonatable groups, such as SDS, was capable of similar effects to those produced by diamines. Fig. 6 illustrates the electropherograms of ovalbumin

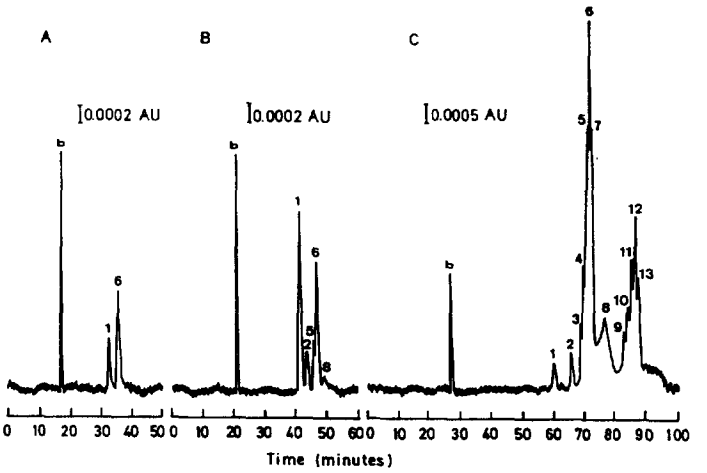

Fig. 4. HPCE separation of ovalbumin. Electrophoresis was carried out in a 69 cm × 75 μm I.D. (63 cm to the detector) capillary in the presence of (A) 0.087, (B) 0.174 or (C) 0.87 m*M* SPD in 25 m*M* borate buffer (pH 9.0). Separation conditions: 11 kV, 25°C. b = Benzene used as neutral marker.

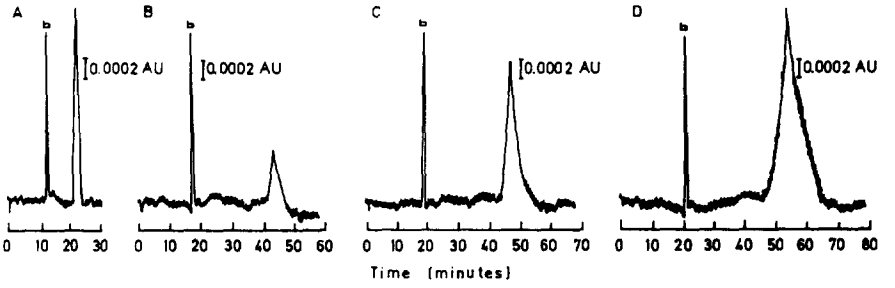

Fig. 5. HPCE separation of BSA. Electrophoresis was carried out in a 69 cm × 75 μm I.D. (63 cm to the detector) capillary (A) in absence or in the presence of (B) 0.087, (C) 0.174 or (D) 0.87 m*M* SPD in 25 m*M* borate buffer (pH 9.0). Separation conditions: 11 kV, 25°C. b = Benzene used as neutral marker.

with SDS at different concentrations below the CMC. The addition of 0.034 m*M* SDS (Fig. 6A) or 0.34 m*M* SDS (Fig. 6B) to the carrier electrolyte leads to a slight change in the electrophoretic profile, although the peak efficiency is very high when we compare these with those obtained in the absence of any modifier (see Fig. 1A). The EOF was maintained, as deduced from the migration time of benzene at 12.1 min, and also the current through the capillary (Table 1). The main peak (No. 6) migrated at about 18 min and the selectivity factors slightly increased to 1.05 and 1.09 in the presence of 0.034 and 0.34 m*M* SDS, respectively. When 7 m*M* SDS was added to 25 m*M* borate buffer (Fig. 6C), the migration time of the ovalbumin peak increased to 31 min (No. 6), although both the EOF and selectivity factor $\alpha_{6,1}$ were similar to those obtained with lower SDS concentrations (Table 1). Nevertheless, a small peak at 38.3 min (No. 12) can be resolved in ovalbumin preparation. The selectivity factor $\alpha_{12,6}$ of 1.23 was very high

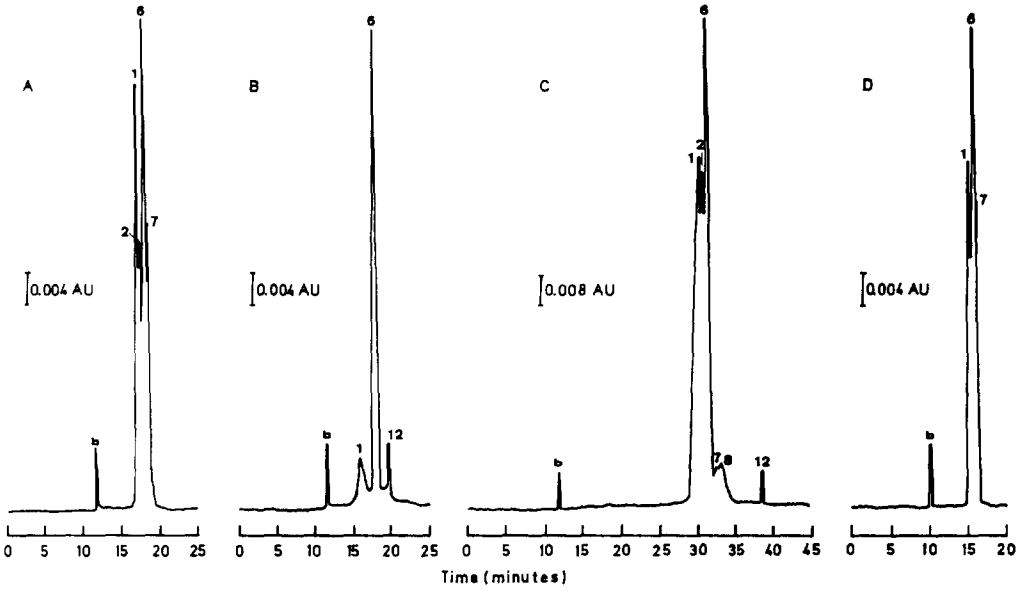

Fig. 6. HPCE separation of ovalbumin. Electrophoresis was carried out in a 69 cm × 75 μm I.D. (63 cm to the detector) capillary in the presence of (A) 0.034, (B) 0.34 or (C) 7 m*M* SDS in 25 m*M* phosphate buffer (pH 9.0); (D), addition of 0.034 m*M* SDS to 2.5 m*M* borate buffer (pH 9.0). Separation conditions: 11 kV, 25°C. b = Benzene used as neutral marker.

compared with that obtained in the presence of amines (Table 1). A decrease in the concentration of the electrolyte buffer to 2.5 m*M* with 0.034 m*M* SDS added did not produce any effect on the resolution of ovalbumin peaks (Fig. 6D and Table 1).

At SDS concentrations below the CMC, monomers of ionic surfactant molecules can act as solubilizing agents for hydrophobic solutes, as ion-pairing reagents or as wall modifiers [18]. In this study, the slight ionic interaction between the charged end of SDS and ovalbumin or the hydrophobic interaction between the alkyl chain of the surfactant and the hydrophobic moieties of the protein was not sufficient to resolve ovalbumin microheterogeneity. Exclusively, a decrease in EOF was observed when 7 m*M* SDS was added to the running buffer as a consequence of surfactant adsorption to the capillary wall. This also led to a decrease in migration times. The control of electroosmotic mobility by addition of surfactants to the background electrolyte is well documented [19–21]. It is effective not only for the suppression of EOF but also for its reversal. Huang et al. [21] reported the separation of a six-component carboxylic acid mixture using tetradecyltrimethylammonium ion, and Kaneta et al. [22] the separation of ten catecholamines by addition of a cationic surfactant. The main apolipoproteins of HDL were separated in an uncoated capillary filled with borax buffer containing 0.1% SDS by Tadey and Purdy [12]. However, Kenndler and Schmidt-Beiwl [23] demonstrated that the addition of 0.05% SDS to the carrier electrolyte had no effect on the separation of conalbumin and ovalbumin. They also observed extremely sharp peaks of both proteins at SDS concentrations of 0.1% and higher in the sample. They explained this apparent high efficiency of the separation system by mobility gradients caused by SDS in the sample, in a similar fashion to the terminating ion in isotachophoresis. In most cases, interactions between SDS and proteins tend to be specific and, depending on the experimental conditions, may or may not alter the expected mobility. Moreover, as SDS binds to the protein, it is important to adjust the concentration of this additive in order to maintain the equilibrium during separation. Therefore, differential SDS binding studies are now in progress in order to exploit the separation of proteins by CZE by increasing the difference in charge-to-mass ratio.

3.5. HPCE of ovalbumin in high-ionic-strength buffer

When 100 m*M* sodium borate buffer (pH 9.0) was used as the carrier electrolyte, the current through the capillary increased dramatically to 89 μA. Under these conditions of analysis, the EOF was reduced and an increase in the migration time of ovoalbumin peaks was observed (Table 2). Fig. 7A shows the electropherogram of an ovalbumin preparation where two main peaks (Nos. 1 and 6) had much higher efficiency than those obtained by using 25 m*M* sodium borate as electrolyte. Lauer and McManigill [3] demonstrated that relatively large amounts of salt compete with protein for adsorption sites. Nevertheless, it is important to note that the use of high-ionic-strength buffers may result in intolerable Joule heat generation when using standard-sized capillaries of I.D. between 75 and 100 μm if heat dissipation is not provided [18]. In our system, forced air surrounding the capillary provides efficient removal of Joule heat, making operation under these conditions feasible.

It was also of interest to determine whether the addition of 0.87 m*M* SPD or 0.34 m*M* SDS to 100 m*M* borate buffer produced the same effect as that with 25 m*M* buffer on the resolution of ovalbumin microheterogeneity. Addition of 0.87 m*M* SPD to 100 m*M* carrier electrolyte permitted nine peaks to be resolved from ovalbumin, four less than those obtained with 25 m*M* borate (see Fig. 4).Decreases in both the migration times and selectivity factors $\alpha_{6,1}$ and $\alpha_{12,6}$ were produced. These results indicate that not only the decrease in EOF resulting from cationic SPD interacting with negative free silanol groups of the capillary wall enhanced the resolution of glycoforms, but also the concentration of the buffer itself in competition with the same groups. The effect of 0.34 m*M* SDS on 100 m*M* borate buffer is more difficult to explain.

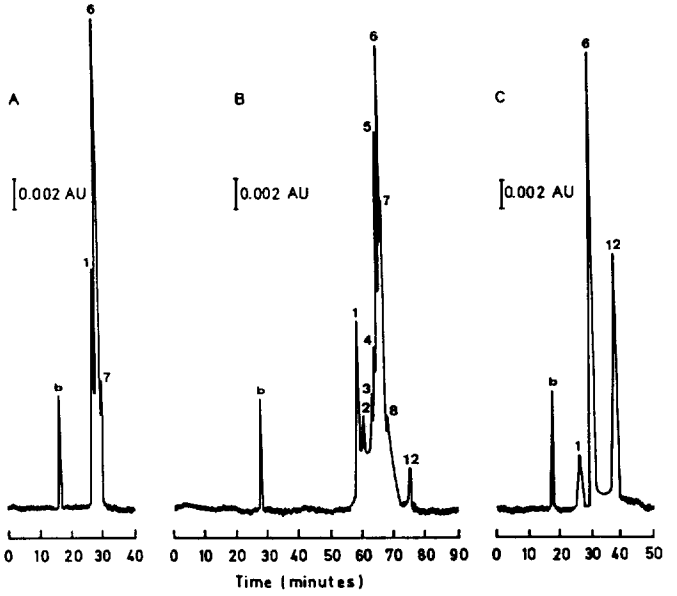

Fig. 7. HPCE separation of ovalbumin. Electrophoresis was carried out in a 69 cm × 75 μm I.D. (63 cm to the detector) capillary (A) in the absence or in the presence of (B) 0.87 m*M* SDP or (C) 0.34 m*M* SDS in 100 m*M* borate buffer (pH 9.0). Separation conditions: 11 kV, 25°C. b = Benzene used as neutral marker.

Fig. 7C shows the electropherogram obtained in the presence of 0.34 m*M* SDS, where three peaks of ovalbumin were resolved (Nos. 1, 6 and 12). The decrease in EOF caused by the effect of a high ionic strength resulted in an increase in the selectivity factors $\alpha_{6,1}$ and $\alpha_{12,6}$ to 1.17 and 1.25, respectively (Table 1). Issaq et al. [24] also reported that resolution was improved with an increase in buffer concentration.

4. Conclusions

4.1. Dependence of retention factors on the nature of the modifier

Various electrokinetic parameters were measured and the results are discussed in order to obtain an overview.

$\alpha_{6,1}$ and t_0

Fig. 8 illustrates typical plots of $\alpha_{6,1}$ and t_0 versus the nature of the monoamine, diamine or PA used as modifier of the carrier electrolyte. As expected, the selectivity factor, $\alpha_{6,1}$, increased with increase in M_r (Fig. 8A), number of methyl or methylene groups (Fig. 8B), ratio of methyl or methylene to amino groups (Fig. 8C) and total length (represented by the number of amino + methylene groups) of the modifier chain (Fig. 8D). These relationships were almost linear if SPM was avoided, since the maximum number of ovalbumin glycoforms was obtained with other polyamines of shorter chain (see Fig. 5). EOF, deduced from the migration time of benzene (t_0), increased linearly with increase in M_r (Fig. 8E), number of methyl or methylene groups (Fig. 8F), ratio of methyl or methylene to amino groups (Fig. 8G) and length of the chain (Fig. 8H). The equations of the straight lines gave correlation coefficients of about 0.95, except that of the ratio of methylene to amino groups, which was 0.85. These results indicate that the resolution between the two main peaks (Nos. 1 and 6) of ovalbumin depends on the magnitude of the cationic chain of the monoamine/diamine/PA used as an additive.

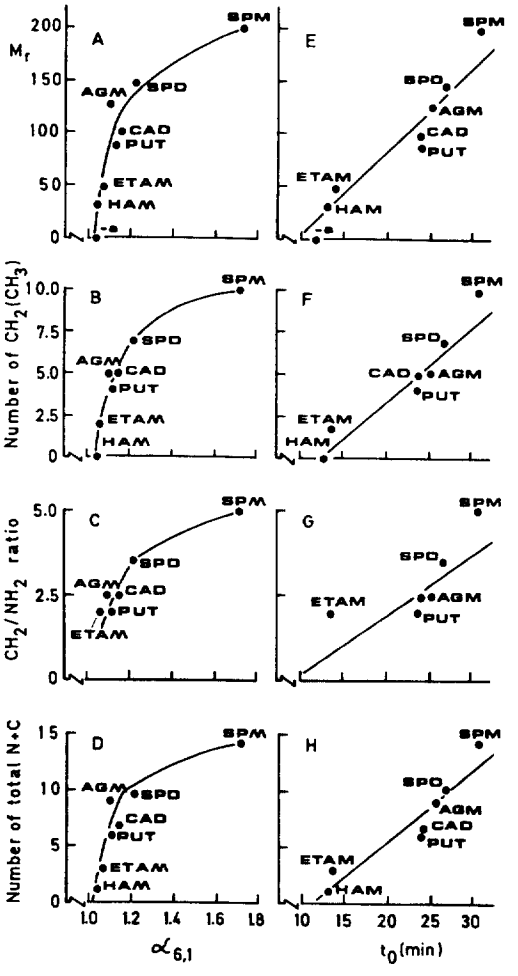

Fig. 8. Selectivity factor, $\alpha_{6,1}$ and t_0 versus M_r (A and E, respectively), number of methyl + methylene groups (B and F, respectively), ratio of methyl or methylene groups to amino groups (C and G, respectively) and number of amino + methylene (or methyl) groups (D and H, respectively) of the main chain of the modifier used as an additive. Abbreviations of modifiers as in Fig. 2; −a indicates the absence of an additive. Equations of the straight lines: (E) $y = 8.041x - 75.994$; $r = 0.95$; (F) $y = 0.439x - 5.266$; $r = 0.95$; (G) $y = 0.187x - 1.755$; $r = 0.85$; (H) $y = 0.602x - 6.516$; $r = 0.95$.

Retention factors, k'_1 and k'_6

Fig. 9 shows the change in k' values of the two main peaks of ovalbumin (Nos. 1 and 6) with the chain length of the additive used in the running buffer. A great increase in the values of both retention factors is observed between two and

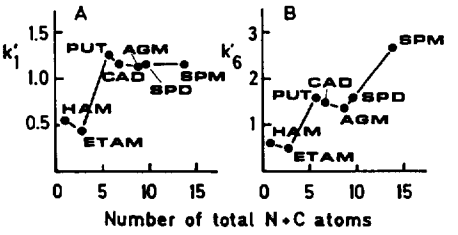

Fig. 9. Retention factors, (A) k'_1 and (B) k'_6, versus the number of amino + methylene or methyl groups of the main chain of the modifier used as an additive. Abbreviations of modifiers as in Fig. 2.

five groups in the cationic chain; from five to fourteen groups, k'_1 remains almost constant (Fig. 9A), whereas k'_6 increases again (Fig. 9B); that is, the retention of peak 6 increases linearly with increasing number of groups of the modifier chain, whereas the retention of peak 1 is independent from a certain length of the additive.

Selectivity factors, $\alpha_{1,0}$, $\alpha_{6,1}$ and $\alpha_{12,6}$, and ovalbumin microheterogeneity

Fig. 10 displays the dependence of the number of peaks detected from ovalbumin preparation on the resolution between the three main peaks (Nos. 1, 6 and 12). The expression of ovalbumin microheterogeneity, which can be translated as the number of peaks detected, increases as the resolution increases. However, there is not the same relationship depending on the selectivity factor. While the number of peaks increases for some similar values of $\alpha_{1,0}$ (Fig. 10A) or $\alpha_{6,1}$ (Fig. 10B), this increment in the number of peaks is more dependent on $\alpha_{12,6}$ (Fig. 10C).

The present study indicates that one may effect the resolution of different glycoforms and resolve protein microheterogeneity by altering the ionic environment inside the capillary and the net charge of the protein. Multiple-buffer additive strategies should therefore be explored to exploit the combinatorial selectivities.

Acknowledgements

The authors thank Professor C. Vicente for his expert assistance in the discussion of the results

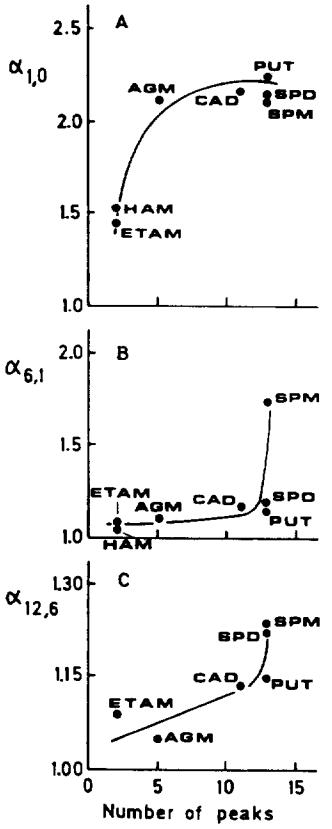

Fig. 10. (A) $\alpha_{1,0}$, (B) $\alpha_{6,1}$ and (C) $\alpha_{12,6}$ versus the number of ovoalbumin peaks resolved in the electropherograms from Figs. 1, 3 and 4. Abbreviations of modifiers as in Fig. 2.

and Miss Raqauel Alonso for technical assistance and acknowledge the support of a grant from the Comunidad Autónoma de Madrid (Spain), No. C243/91.

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