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Separation of proteins and peptides by capillary electrophoresis in acid buffers containing high concentrations of surfactants

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Abstract

Separations of proteins at acid pH in the presence of a high concentration of surfactant [sodium laurylsulfate (SDS), 50 mmol/l] was investigated. The purpose of using high concentrations of SDS as background electrolyte modifier was threefold: First, the surfactant exerts a washing effect upon the capillary wall thus preventing binding of analytes and possible clogging of the capillary. Second, it was revealed that even under very acid conditions (below pH 3) the surfactant is capable of forming associates with protein analytes which still bear considerable negative charge and can be separated on this basis. Third, the system can be applied not only for protein mixtures sufficiently soluble in neutral to alkaline media (leukocyte lysates, standard proteins), but it can be used also with proteins, that are under such conditions virtually insoluble and their solubilization is possible in acid buffers only (eggshell proteins or collagen CNBr fragments). The result was that adsorption to the capillary wall was minimized and the analytes were separated as negatively charged associates with high efficiency. With collagen fragments partition was possible on the affinity differences of the peptides to the surfactant micelles and inner wall of the capillary. Theoretical plate counts approaching 100 000 were easily achieved even with proteins which under the more conventional operation conditions exhibit considerable sticking to the capillary wall. The other feature of this system is that the associates move very rapidly to the anode. Owing to the low pH, endoosmotic flow is negligible, and therefore the system has to be operated at reversed polarity.

Keywords: Buffer composition; Proteins; Peptides; Collagen

1. Introduction

Since the first report by Terabe et al. [1] micellar electrokinetic chromatography proved a useful separation technique for a number of compounds differing considerably in their chemical structure. Rather recent investigation showed that the micellar ionic solutions can also be successfully applied for the separation of complex protein mixtures ([2]; for reviews see [3–6]) because the mobility of charged micelles is greater then any of the protein-micelle complexes [2]. Generally protein migration times were shown to increase with increased micelle [sodium laurylsulfate (SDS)] concentration in the background buffer [7], an effect that was used for the separation of genetically engineered protein from fermentation broth. Most of these separations were run at alkaline pHs (8–9), using typically borate buffers, occasionally with an organic modifier (acetonitrile) to modulate the partition of analytes followed between the aqueous and the micellar phase [8–13]. With decreasing pH of the background electrolyte, the endoosmotic flow slows down while the movement of charged micelles towards anode (in the case of SDS) remains quite fast. This results in the migration time increase which finally could be

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such that working in the reversed polarity mode is necessary as shown in Refs. [8,14]. The sequence of emerging proteins remains unchanged even at low SDS concentration (0.1%) except collagen fragments [14] where the sequence of polypeptides (ranging in molecular mass from 1000 to 60 000 rel. mol. mass units) is a mirror image to the profile obtained at e.g. pH 9.0. This was ascribed to the high internal homogeneity of the collagen fragments and their strong affinity to the bare silica capillary wall. To our best knowledge the lowest pH at which such separations were carried out was 4.5 [8] except a recent work of Quirino et al. [15] where electrophoresis at acid pH (2.0) in 100 mmol/l phosphate buffer containing 50 mmol/l SDS was used along with a water plug for sample stacking for the separation of alkyl phenyl ketones (propionophenone, hexanophenone and valerophenone) and polycyclic aromatic hydrocarbons (phenanthrene, fluorene and naphthalene). The question one is tempted to ask is whether or not separations at low pH values can be done, in particular with proteins that are difficult to solubilize in alkaline buffers, Such a system is expected to be run in the reversed polarity mode (detector to anode) as it is known that at pH below 5 the flow of SDS micelles is reversed [16].

2. Experimental

2.1. Capillary electrophoresis

All runs were done with Beckman PACE instrument system 5500 (Beckman, Fullerton, CA, USA). Bare fused silica capillary of 57 cm (50 cm to the detector)×75 μm I.D. was used. Detection was done by UV absorbance recording at 214 nm. Before analysis the capillary was washed for 2 min with the background electrolyte. Sample injection was done hydrodynamically (3.45 kPa overpressure, applied for 2 s). Next 1 kV voltage was applied (twice for 0.5 min) using two different jars for rinsing the electrodes. Every separation was run at 35°C. After the separation came to its end, the capillary was washed step-wise with the background electrolyte (2 min), water (2 min), 1 mol/l NaOH (5 min), water (2 min), 3 mol/l HCl (10 min), and water (2 min). Before analysis the background electrolyte was filtered using Gelman Acrodisc PTFE filter, 0.45 μm. Since the proteins associated with SDS move towards the anode rather fast and since the endoosmotic flow at pH 2.5 is minimal indeed, the system has to be run in reversed polarity mode.

2.2. Chemicals used

Sodium dihydrogenphosphate and hydrochloric acid were products of Lachema Brno, Czech Republic, and were of analytical-reagent grade. SDS, 2-mercaptoethanol, bromcyan (cyanogen bromide) and ethylenediaminetetraacetic acid disodium salt (EDTA, Titriplex III) was from Merck (Darmstadt, Germany), ammonium hydrogencarbonate was from Sigma (St. Louis, MO, USA) and formic acid from Fluka (Buchs, Switzerland). All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA).

2.3. Model mixtures

To show the applicability of SDS containing buffers at acid pH, four sets of model mixtures were selected.

(a) A set of standard proteins composed of α-lactalbumin, carbonic anhydrase, urease (all Sigma) and cytochrome *c* (Reanal, Budapest, Hungary). These proteins were dissolved in 5% formic acid in concentration 250 μg/ml (α-lactalbumin, cytochrome *c*), 125 μg/ml (carbonic anhydrase) or 750 μg/ml (urease).

(b) A set of peptides obtained by CNBr cleavage of rat tail tendon collagen. This sample contained fragments of both type I and III collagen and was prepared by the procedure described in our previous communication [17], based on the classical paper of Scott and Veis [18]. Sample was solubilized in 5% formic acid (1.5 mg/ml); in this case injection was only 1 s.

(c) Commercial immunomodulator, essentially a leucocyte lysate ($2 \cdot 10^8$ cells per ampule), available under the name of Immodin from Sevac, Prague, Czech Republic (containing Lawrence Transfer factor, Ref. [19]) aside to a number of proteins with unclear (if any) pharmacological activity.

(d) A set of CNBr peptides was obtained from insoluble egg-shell proteins.

Whole eggs were washed with water and methanol and were treated by 5% EDTA (pH 7.6) containing 10 mmol/l 2-mercaptoethanol (three times the egg volume) for 60 min at room temperature. The insoluble organic layer resulting on the egg surface after this partial decalcification was scratched and accumulated. This resulted in the removal of the outer eggshell layer (the cuticle). The eggs were then treated with 0.6 mol/l EDTA (pH 7.6) containing 10 mmol/l 2-mercaptoethanol (three times the egg volume) for 90 min at laboratory temperature. This resulted in the decalcification of the palisade layer (the main calcified layer of the eggshell). The eggs were scratched, the material accumulated by washing with water and centrifugation (1000 *g*, 15 min). The resulting pellet was resuspended in water and centrifuged at the above conditions (repeated three times) and then lyophilized. This procedure avoided contamination of the proteins analysed with proteins present in the mammilary cores and the inner surface of the eggshell. In other words, proteins analysed originated from the partial (outer surface, palisade layer) decalcification of the eggshell (consequently further decalcification of the bottom layer of the palisade layer and mammilary cores is possible). For review about structure of eggshell see Ref. [21].

CNBr peptides was prepared by the slightly modified procedure used for collagen (see above). Briefly, methionyl residues in peptides were reduced by 25% 2-mercaptoethanol in 0.2 mol/l ammonium hydrogencarbonate overnight. The proteins were lyophilized and cleaved subsequently three times by CNBr in 70% formic acid (70 mg of CNBr and 4 ml of formic acid per 10 mg sample) under N_2 overnight. At the end of the incubation, the solution was diluted with 10 volumes of water and lyophilized. Before analysis the samples were reconstructed in 5% formic acid (2 mg/ml).

It should be noted that of these four model protein mixtures a and c can be solubilized over a wide range of pH (preferably neutral or alkaline) while sets b and d are virtually insoluble at alkaline pH. For set b solubilization in at least 0.5% acetic acid is needed while set d can be solubilized in 2% formic acid only. Samples for analysis were prepared in the run buffer (sets a and c), collagen peptides and egg-shell proteins were solubilized in 5% formic acid at concentrations as specified in the text.

2.4. HPLC

Separation of collagen CNBr peptides was done as described before [20].

Separation of CNBr peptides obtained from insoluble egg-shell proteins was done by the HP 1100 system (Hewlett-Packard, Palo Alto, CA, USA) consisting of a degasser, binary pump, autosampler, thermostatted column compartment and diode array detector. The column used was Zorbax 300SB-C_8, narrow-bore, 2.1×150 mm, 5 μm (Rockland Technologies, USA). Elution was done by a linear gradient using mobile phase A (water with 1% formic acid) and B (water–acetonitrile, 20:80, v/v, with 0.85% formic acid). Elution started with isocratic elution with solvent A (2 min), followed by a gradient to 20% B at 8 min and another gradient to 100% B (25 min). Next the column was equilibrated for 10 min with solution A before the next run. The flow-rate was 0.20 ml/min, column temperature was held at 30°C. UV detection was done at 214 nm and injection volume was 5 μl.

3. Results and discussion

The results of the separation of the standard mixture of α-lactalbumin, cytochrome *c*, carbonic anhydrase and urease showed a reasonable separation of all compounds except cytochrome *c* and carbonic anhydrase which overlapped. The tailing part of the carbonic anhydrase is apparently a contaminant present in this enzyme preparation (Fig. 1). Urease being the most polar protein apparently associated least with the surfactant and moved at the end of the electropherogram while proteins with medium to low polarity were more prone to association with the detergent and moved more rapidly towards the anode as expected. In general, the sequence of the peaks reflects the hydrophobicity of analytes involved, the most apolar being eluted first. Numbers of theoretical plates obtained (in order of increasing migration time) were $319 \cdot 10^3$, $82 \cdot 10^3$, $120 \cdot 10^3$ and $60 \cdot 10^3$ respectively. It is obvious at first glance that the system offers a large separation window for proteins

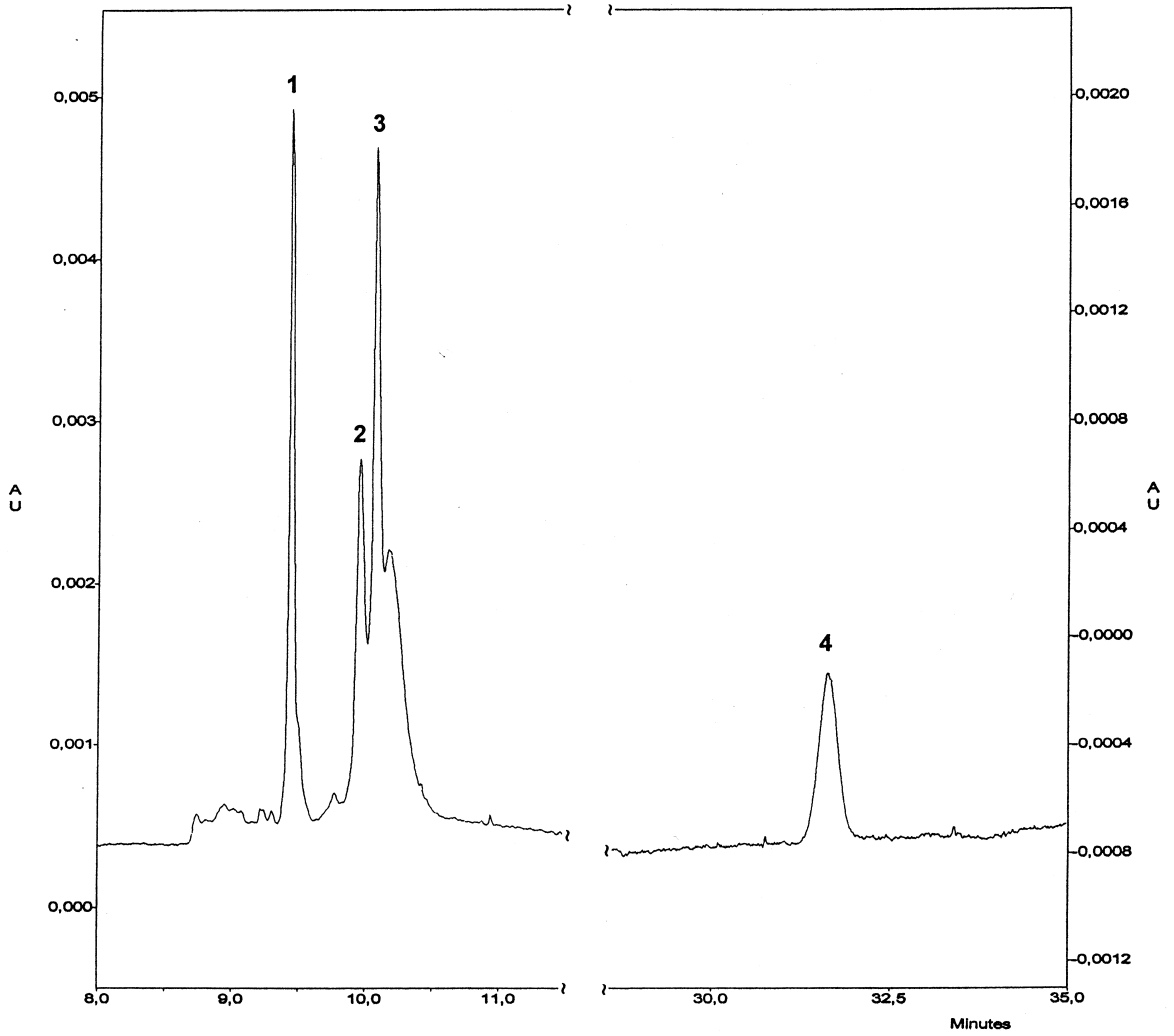

Fig. 1. Separation of four standard proteins at pH 15 kV, reversed polarity mode; 50 mmol phosphate buffer (pH 2.5 adjusted with hydrochloric acid) containing 50 mmol/l SDS. Peaks: 1=α-lactalbumin; 2=cytochrome *c*; 3=carbonic anhydrase (the shoulder on the descending part of the peak is an impurity present in this enzyme), 4=urease.

of medium polarity (about 20 min of migration time) between the carbonic anhydrase and urease peaks. It can be expected that such proteins are abundant in the cells. Analysis of the immodin preparation confirmed this assumption (Fig. 2). Reproducibility of the pattern obtained with this sample was excellent as shown in Fig. 3 where three runs done within a nine hour period are shown. Note the shift of a late peak indicated by the arrow which moves towards the anode with increased exposure to room temperature (finally it fuses with the large peak emerging at 28.6 min). This shift was perfectly reproducible in three sets of experiments done within consecutive days. It is assumed that the reason for the mobility change of this peak is the instability of the protein in the sample (speculation of its degradation at room temperature). Note also that even the reproducibility of the very small peaks on the electropherogram is also at least acceptable. It is to be stressed that in preliminary experiments done in both the treated and untreated capillaries at high pH (~8-9) only a single peak without any indication of further separation was

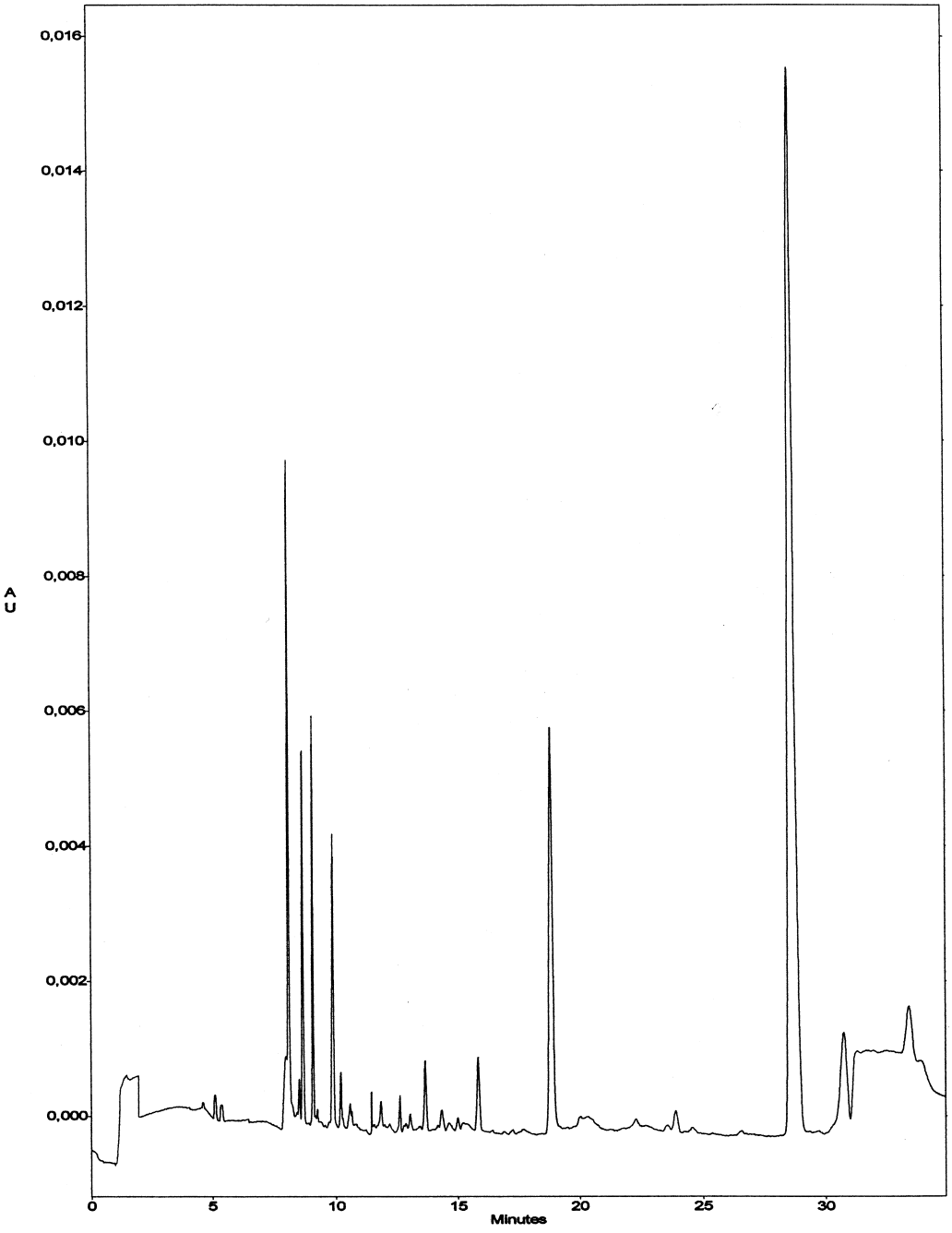

Fig. 2. Separation of leucocyte lysate preparation (immodin, Sevac, Prague, CZ). Separation conditions as in Fig. 1, except that the voltage was increased to 20 kV after 30 min. Reversed polarity mode.

revealed. The baseline increase after 30 min of running time is caused by an increase of voltage applied. The run was done with sudden voltage change. At the beginning 15 kV were applied followed by an increase to 20 kV at 30 min in order to shorten the runs.

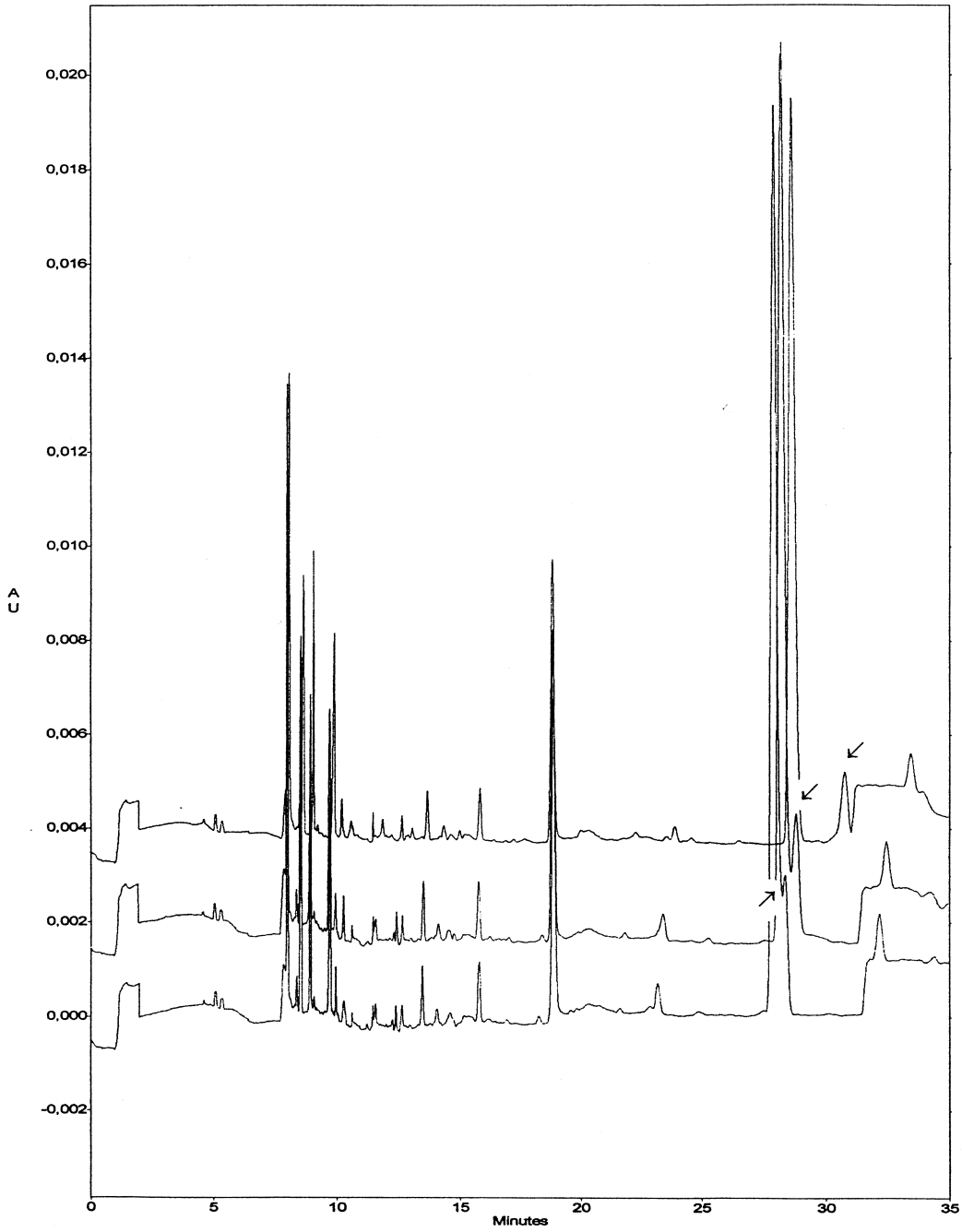

Fig. 3. Separation of the leucocyte lysate under conditions specified in Fig. 2. The set of runs presented shows reproducibility of the profile. Arrows indicate a compound unstable at laboratory temperature which shifts towards shorter migration times upon standing. Upper recording-first experiment. Medium recording-3rd run after 4.5 h of sample standing at room temperature. Bottom recording-6th run after nine hours standing at room temperature.

The separation of CNBr peptides released from the insoluble egg-shell proteins is shown in Fig. 4. The insert presents the whole separation indicating that a considerable proportion of the fragments present in the sample is rather polar and is only poorly associated with the detergent and therefore also less charged; consequently in the reversed polarity mode they come last before the detector's window. The enlarged front part of the electropherogram shows peaks strongly associated with the detergent (peak at the fifth minute is always present in samples treated by cyanogen bromide and should not be considered). Reproducibility of this part of the electropherogram shown in the main part of Fig. 4 is similar to that presented in Fig. 3 (data not shown). In Fig. 5 the separation of the same mixture of egg-shell protein fragments by reversed-phase chromatography is shown for comparison. If one limits oneself to the dominant peaks only, the overall profile in micellar electrokinetic chromatography (MEKC) and RP-HPLC appears reversed which supports the idea that while most hydrophobic peaks are most retained in reversed-phase chromatography and appear at the end of the chromatogram, in capillary electrophoresis carried out in the presence of the surfactant at acid pH, the most hydrophobic species have the highest affinity to the surfactant (being most associated with SDS micelles) and, consequently, move in the front of the electropherogram (in the reversed polarity operational mode). On the other hand, the overall profile in SDS-electrophoresis is different in details to the reversed-phase separation; it is therefore possible to conclude that the selectivity of both systems is different and no detailed comparison is possible. On the other hand this comparison says that although both systems exploit the hydrophobic properties of the analytes separated, their combination is likely to add to our knowledge about the composition of this complex mixture (individual peaks are marked only tentatively as no information about their nature is currently available). Note, however, that peaks Nos. 3 and 4 move in a reversed order to that expected (as verified with isolated fractions). This suggests that the order in which individual peptides emerge from either system (i.e. HPLC and CE) is probably also influenced by other factors than by the analyte's hydrophobicity. Typically, one can speculate about sterical hindrances, e.g. if a particular hydrophobic domain in the sequence is sandwiched between two polar regions it may not be easily recognized by either the C_{18} moiety or SDS yielding, thereby, differences between the reversed-phase and MEKC profiles. It is to be noted that separation of eggshell proteins CNBr fragments completely failed if acid pH buffers (without the detergent) were used. Detailed characterization of the CNBr peptides of insoluble eggshell proteins is the subject of another communication. A considerable problem with analysing eggshell proteins and their fragments is the limited knowledge about their composition caused by their extreme insolubility (the soluble part of the eggshell proteins is known to contain a number of proteins, including proteoglycans and ovalbumin, see Refs. [21–23]).

The separation of collagen CNBr fragments seems a bit more complex (Fig. 6). In HPLC these fragments elute in an order that corresponds to the number of hydrophobic domains contained in the individual members of the set. Because the larger peptides (owing to high internal homogeneity of the parent protein) contain more hydrophobic regions, the result of HPLC separation is that the small peptides elute first, the large one last, allowing an easy determination of the molecular mass of a particular fragment [20]. In the capillary electrophoresis run at very acid pH (~2.5) the result is very similar: smaller peptides run fast, the larger one are more slow and the overall profile is quite similar to that seen in HPLC. It was therefore hypothesized that the partition mechanism here reflects interaction (adsorption) of the individual peptides to the capillary wall [17] and their elution by the passing by buffer. A supporting evidence for this conclusion was drawn from an experiment with surface treated capillary in which the separation was virtually lost [17]. If the separation is carried out at low (submicellar) concentration of the surfactant (at pH 4.5) the profiles obtained are apparently not only derived from the attachment of individual peptides to the capillary wall, but the peptides apparently associate with the detergent molecules causing the selectivity of the system to be increased [14]. If the concentration of the surfactant is increased to supra micellar concentration, association of the adsorbed peptides occurs with surfactant clusters (micelles), which results in more easy detachment and faster

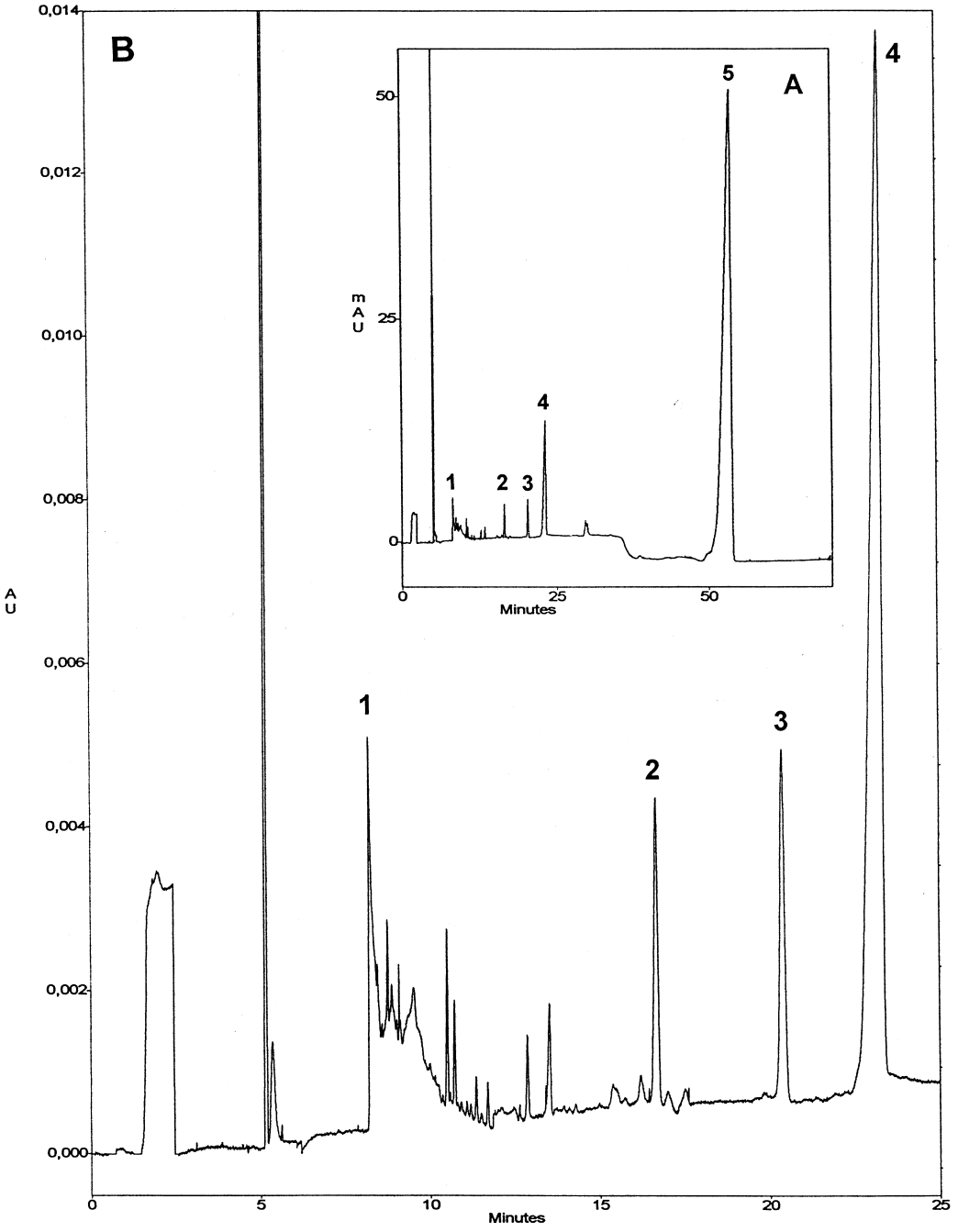

Fig. 4. Separation of CNBr fragments of egg-shell proteins. Conditions as described in the legend to Fig. 1. Inset (A): whole separation indicating the presence of a large fraction associating poorly with the surfactant. Main figure (B): enlarged front section of the electropherogram showing the peaks of protein fragments associating easily with the surfactant emerging within the first 25 min of running time. The sharp peak at five minutes is an artefact present in samples treated with CNBr.

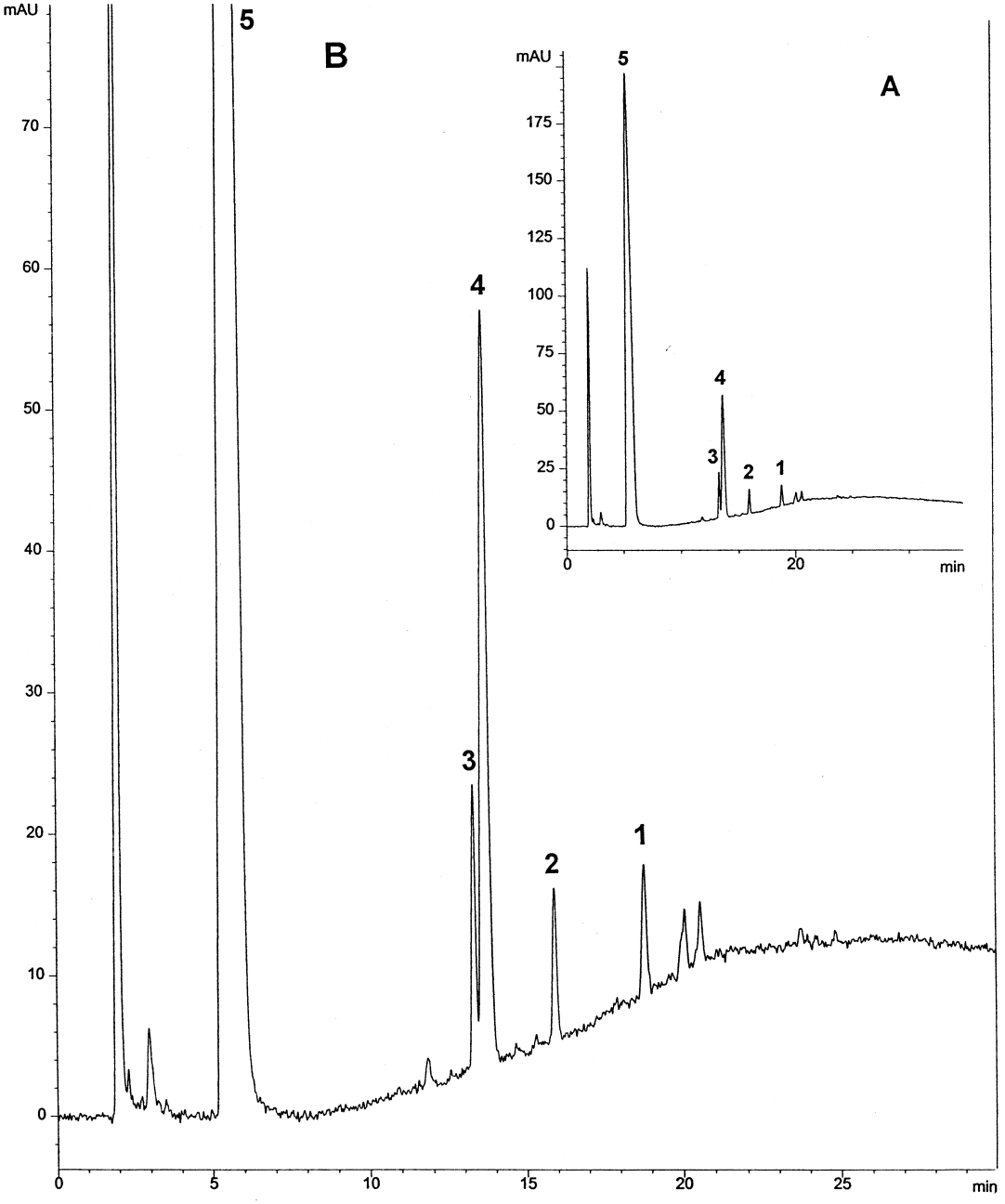

Fig. 5. Reversed-phase chromatography of the same sample shown in Fig. 4. Note the reversed order of the dominant peaks (corresponding peaks are indicated by the same numbers). Inset (A): whole separation, the main figure (B): enlarged chromatogram. For chromatographic conditions see Experimental.

anodic movement, however the selectivity is worse. In systems using either sub or supramicellar concentration of the detergent the separation is more rugged and less dependent on the amount of sample injected (in the absence of surfactant clogging the capillary which frequently occurs). Also, as the

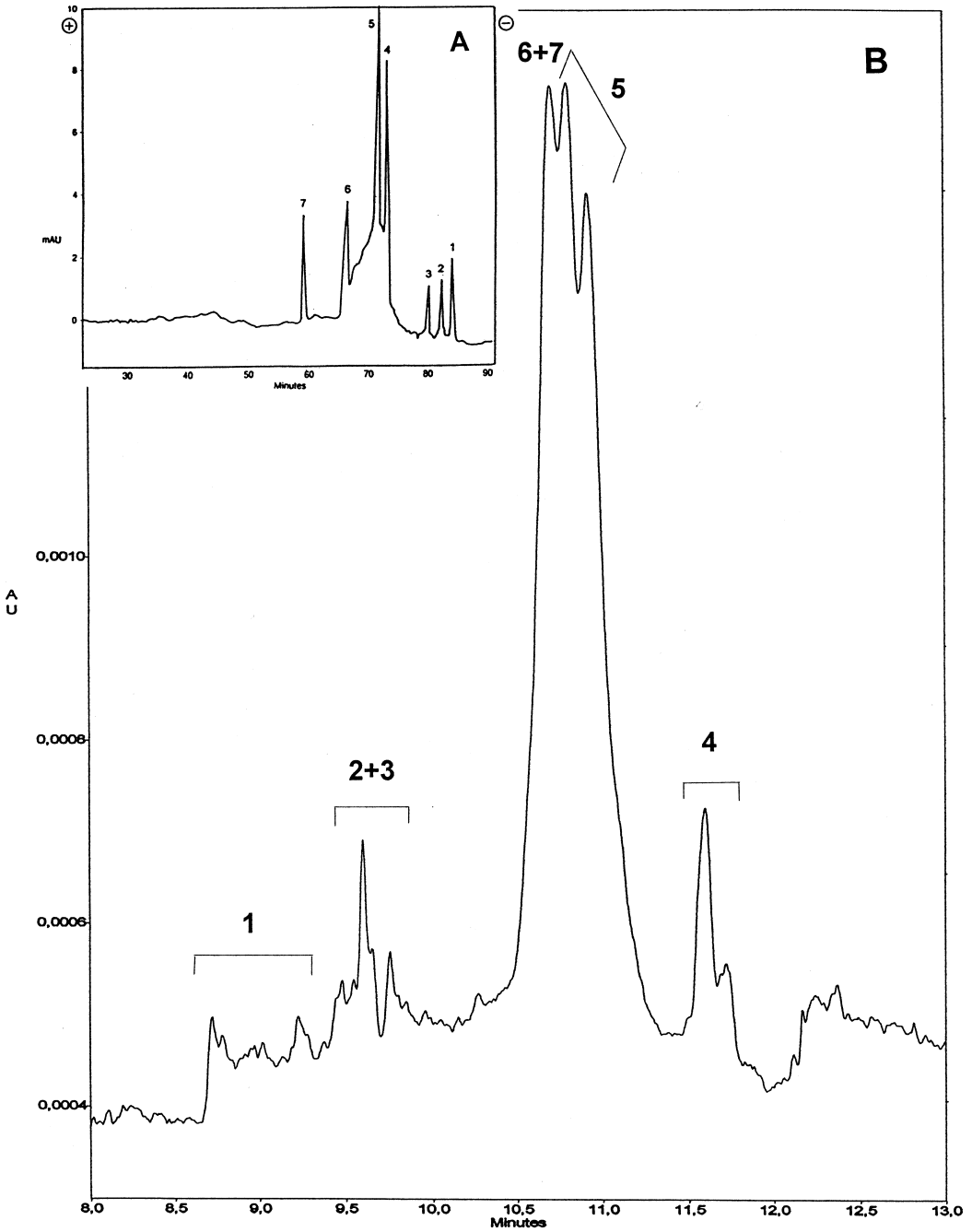

Fig. 6. Separation of rat tail tendon collagen CNBr peptides in the presence of 50 mmol/l SDS in the background electrolyte (separation conditions as in Fig. 1). Insert: the same sample run in submicellar detergent concentration (0.1%≈3.5 mmol/l SDS). Both runs in reversed polarity mode. Note changes in the peak sequence when high surfactant concentration in the background electrolyte is used (insert taken from our previous report, see Ref. [14]). Peaks: (A) 1=α_1(I)CB$_2$, 2=α_1(I)CB$_4$, 3=α_1(III)CB$_4$, 4=$\alpha_{.1}$(I)CB$_6$, 5=α_1(I)CB$_7$+α_1(I)CB$_8$, 6=α_2(I)CB$_4$, 7=α_2(I)CB$_{3,5}$+[α_1(III)CB$_9$]$_3$ (B) 1=α_1(I)CB$_2$, precise location unknown; 2-4 as in A; 5=contains α_1(I)CB$_7$+α_1(I)CB$_8$+ uncompletely cleaved products, 6 and 7 as in A. None of the peaks shown in B represents a pure peptide except No. 4 [α_1(I)CB$_6$].

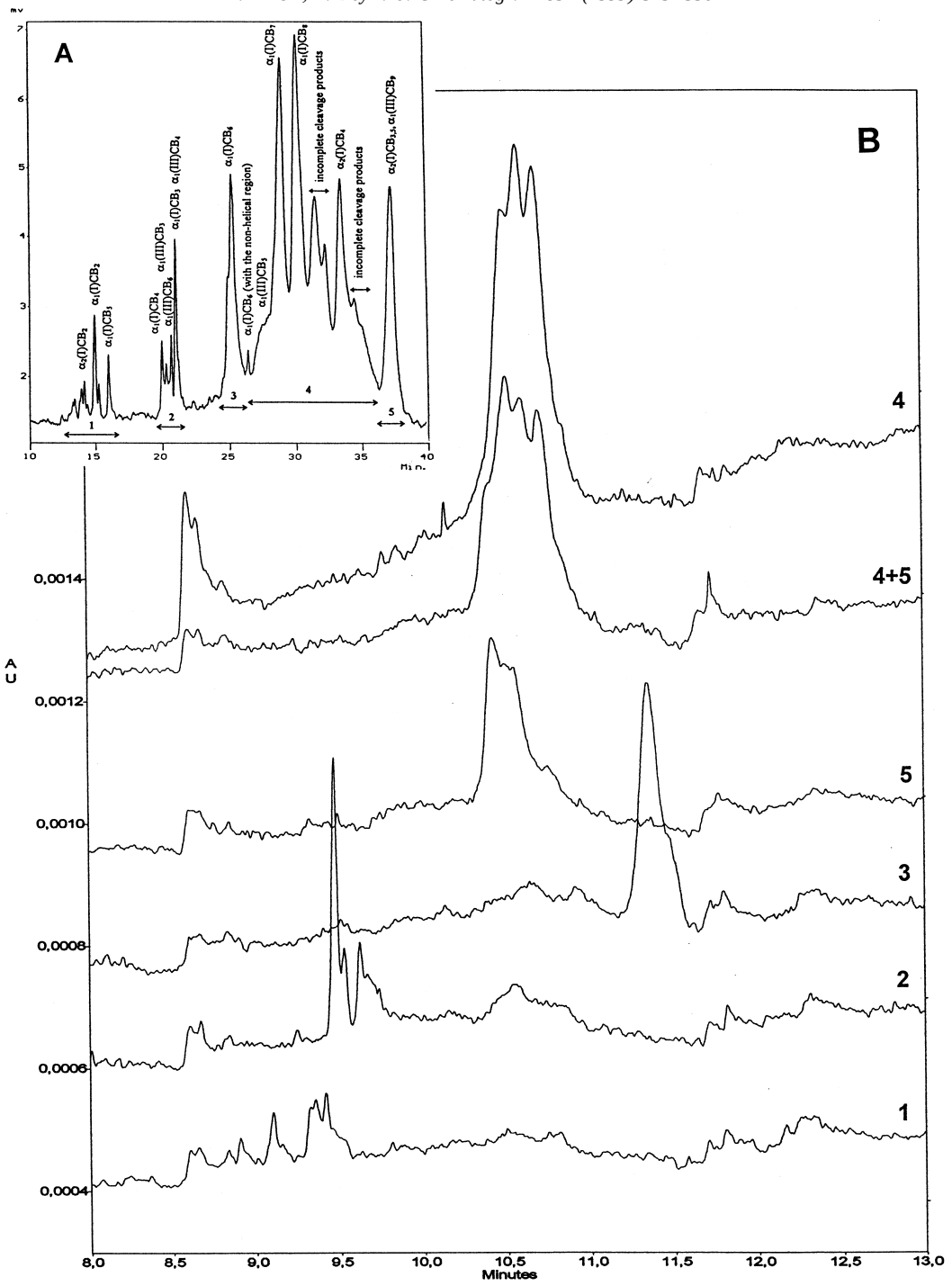

Fig. 7. Identification of CNBr collagen fragments in the electrophoretic profile obtained at high concentration (50 mmol/l) of the detergent in the background electrolyte. (A) The result of reversed-phase chromatography of the sample run under conditions as specified in Ref. [20]. Accumulated peptide fractions (see horizontal bars) were separated by capillary electrophoresis. (B) The resulting pattern indicates that small peptides are displaced to the front of the electropherogram (compare inset to Fig. 6). Peptides present in the chromatographic fractions 3, 4 and 5 move in the expected order, i.e. the larger ones move more rapidly to anode than the smaller ones.

anodic movement of individual peptides in systems exploiting SDS in the background electrolyte is quite fast, electrophoretic separations must be run with reversed polarity. However it has to be stressed that in the procedure in which supramicellar concentration of the detergent was used, association of the peptides may not strictly follow the number of hydrophobic domains in the molecule (e.g. for sterical reasons as mentioned above). Another reason could be that the peptide may be simply too small to contain distinct hydrophobic domains. Consequently the sequence of emerging peaks would not strictly follow the number of such domains and the overall pattern cannot be interpreted in terms of the molecular mass of the analyte as some of the peaks will be displaced as shown in Figs. 6 and 7.

4. Conclusion

Capillary electrophoretic separation at low pH in the presence of surfactants (SDS) was shown to offer additional possibilities in separating protein analytes. At high (supramicellar) concentrations the proteins associate with the surfactant micelles and move rapidly to the anodic end of the capillary. Consequently, the separations have to be done in the reversed polarity mode. As the endoosmotic flow under the given conditions is very slow, the separation is ruled in practice only by the charge of the arising aggregates provided that no strong (hydrophobic) interactions between the proteins to be separated and inner surface of the capillary take place. In the latter case an additional partition mechanism comes into the action, involving both the affinity of the analyte to the capillary wall and to the surfactant micelles. This seems to be sterically affected as demonstrated in the separation of collagen derived CNBr peptides. Besides the possibility to separate protein analytes soluble only in acid media, the background electrolyte exerts simultaneously a washing effect upon the capillary preventing it from clogging if large protein samples are applied [24]. Practical application to leucocyte lysates and eggshell protein is shown aside to the separation of protein standards. Comparison of published data on the separation of collagen derived CNBr fragments (as a model set) by capillary zone electrophoresis in the absence of the detergent, by electrophoresis at sub and supramicellar detergent concentration and RP-HPLC allowed postulation of the partition mechanisms involved.

Acknowledgements

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