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Comparison of different electrokinetic separation modes applicable to a model peptide mixture (collagen type I and III CNBr fragments)

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Abstract

A number of electromigration separation modes were applied to the separation of CNBr-released peptides from rat tail tendon collagen (microemulsion electrokinetic chromatography, methanol- or ethanol-modified background electrolytes and the separation in the presence of molecular sieving effect exerting polymer, both in the presence and absence of SDS). Electrodriven separations with a Hypersil C₈ packed capillary were investigated as well. The best separations were obtained with either the molecular sieving effect exerting polymer (polyethylene oxide) in the background electrolyte (whether SDS was present or absent) or with the electrodriven chromatography using the C₈ reversed-phase packed capillary. In the latter separation system, it was possible to separate 25–27 peaks of the theoretically expected 24 peptides in the analyzed mixture of which 17 were at least tentatively identified. The additional peaks apparently stem from the incomplete cleavage of the parent collagen α chains. Successful separations can be done either with predominating molecular sieving or hydrophobic partitioning mechanism. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Peptides; Cyanogen bromide; Collagen

1. Introduction

Collagens represent the most ubiquitous proteins of the vertebrate body [1]. Connective tissue contains mainly two types of these proteins, namely collagens type I and III [2,3]. These collagen types can be isolated from the tissue by a rather tedious, at least three-step procedure, which is unsuitable (or at least inconvenient) for estimating their content (or ratio) in the tissue [4–7]. These problems can be overcome by CNBr cleavage of the proteins involved [8]. In this way the protein fragments are brought into

solution, however, the mixture is quite complex (24 peptides can be theoretically released from a collagen type I and III mixture, occurring in natural tendon or skin collagen samples, see Ref. [9]). Both gel electrophoresis and recently capillary electrophoresis (CE) have not yet offered a complete separation of these fragments, though quantitation of individual collagens in tissues is possible by CE exploiting marker peptides, namely $\alpha_1(\text{I})\text{CB}_2 + \alpha_1(\text{I})\text{CB}_4$ and $\alpha_1(\text{III})\text{CB}_2$ for each of these two collagen types. An equimolar mixture of $\alpha_1(\text{I})\text{CB}_2 + \alpha_1(\text{I})\text{CB}_4$ is used as an internal standard for type I collagen, while $\alpha_1(\text{III})\text{CB}_2$ peptide is used for estimating the proportion of type III collagen [10,11].

As reported previously [10] partial separation of

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collagen type I and III CNBr peptides can be achieved by CE using 25 mmol/l phosphate at pH 2.5 without any modifier of the background electrolyte in the positive mode at 10 kV. Addition of 1% of carboxymethylcellulose as molecular sieving modifier had little, if any effect. By this approach the above mentioned marker peptides can be quantitated and the proportion of collagens in a tissue sample can be estimated [11]. However the separation suffers from poor resolution of medium-size peptides, typically $\alpha_1(\text{I})\text{CB}_6$, $\alpha_1(\text{I})\text{CB}_7$, $\alpha_1(\text{I})\text{CB}_8$ and $\alpha_2(\text{I})\text{CB}_4$ possessing relative molecular masses (M_r) of 16 500, 24 000, 24 000 and 29 000, respectively. Separation of the marker peptides in 25 mmol/l phosphate buffer, pH 4.5 with 4.5 mmol/l sodium dodecyl sulfate (SDS) is also possible. In this case the separation was carried out at 15 kV per capillary in the negative mode and yielded partial separation of $\alpha_1(\text{I})\text{CB}_6$, $\alpha_1(\text{I})\text{CB}_7 + \alpha_1(\text{I})\text{CB}_8$ and $\alpha_2(\text{I})\text{CB}_4$ peptides, however the time needed for separation exceeded 60 min. The small peptides, typically $\alpha_1(\text{I})\text{CB}_2$, $\alpha_1(\text{I})\text{CB}_4$ and $\alpha_1(\text{III})\text{CB}_2$ were clearly separated, however, the separation of larger peptides was incomplete. In the latter case individual peptides occur at the detector's window in a reversed order compared to the positive separation mode [10]. At pH 2.5 in a 50 mmol/l phosphate buffer (without SDS) the separation is rather fast (around 25 min needed for completion), however only the peptide $\alpha_1(\text{I})\text{CB}_6$ and a mixed peak of $\alpha_1(\text{I})\text{CB}_{3,5} + [\alpha_1(\text{III})\text{CB}_9]_3$ in addition to the small marker peptides were clearly separated.

Based on the fact that the main partition mechanism involved reflects the particular peptide hydrophobicity (the larger peptides owing to the high internal homogeneity of this category of proteins, possess more and/or larger hydrophobic domains than the shorter ones), it appeared reasonable to investigate the hydrophobic properties of the separation system and, if possible, to combine them with a molecular sieving mechanism that may synergistically influence the separation yielding better selectivity. However, it is necessary to emphasize that the separation of the peptides involved comprises apparently multimode separation mechanisms of which some type of partition can predominate depending on the experimental arrangement.

2. Experimental

A Hewlett-Packard Model HP^{3D} CE instrument (Waldbronn, Germany) was used in the experiments described. UV detection was done at 214 nm and the separations were run in both positive or negative mode (anode or cathode connected to the sampling end of the capillary) as described for the individual categories of separations. For electrochromatography both ends of the instrument were pressurized by 10 bar during the analysis. The capillary-containing cassette was thermostated at 20°C. Samples were introduced by overpressure (3.45 kPa) applied for 2–10 s depending on sample concentration.

For electrophoretic separations bare fused-silica capillary of 57 cm (50 cm to the detector) \times 75 μm I.D. was used. Before analysis the capillary was conditioned in the following way: 2 min with 0.1 mol/l NaOH, 2 min with water and 2 min with the run buffer. Next 1 kV was applied (twice for 0.5 min) using two different vials for rinsing the electrodes in a similar way as described in our previous report [12]. Separations were run at 10 kV if not specified otherwise. Most of the runs were done in the negative mode; if the positive mode was used the fact is indicated in the text. Also the composition of run buffers is specified in Results and Discussion and in individual figures.

After the separation was brought to its end the capillary was washed with the background electrolyte (2 min), water (2 min), 1 mol/l NaOH (5 min) and water (2 min). All samples, washing solutions and electrolytes were filtered using an Acrodisc PTFE filter (0.45 μm).

2.1. Chemicals and CEC columns used

Sodium dihydrogenphosphate, boric acid and sodium hydroxide were products of analytical grade purchased from Lachema (Brno, Czech Republic). SDS, 2-mercaptoethanol, and bromocyan (cyanogen bromide) were from Merck (Darmstadt, Germany). Formic acid was a product of Fluka (Buchs, Switzerland). Acetonitrile, ultrapure gradient grade was obtained from J.T. Baker (Deventer, The Netherlands), triethylamine was a product of Sigma (Deishofen, Germany) and orthophosphoric acid was

purchased from Fluka (Deishofen, Germany). Tris was a product of Merck. Polyethylene oxide used for molecular sieving experiments was purchased from Sigma–Aldrich (Prague, Czech Republic), average M_r $4 \cdot 10^4$. All solutions were prepared in Milli-Q water.

The CEC column (250 mm \times 100 μ m) packed with C_8 reversed-phase was a product of Hewlett-Packard (Hypersil C_8). The packing had a nominal particle size of 3 μ m, total length of the column 335 mm, packed length 225 mm. Before use the column was conditioned with acetonitrile–25 mmol/l Tris, pH 8 (80:20) according to the manufacturer's instructions. The inlet of the capillary was checked every three runs for polyimide coating clog which was removed by burning if necessary.

2.2. Model peptides

A set of model peptides was obtained by cyanogen bromide cleavage of rat tail tendon as described in our previous communication [11]. This sample contained both collagen type I and III fragments and the cleavage procedure basically followed that of Scott and Veis [13] in the modifications specified in our previous report (for collagen CNBr-released peptides nomenclature see Refs. [8,13,14]). Briefly, the number attached to each peptide at the end reflects the position of a particular fragment in the elution profile obtained after CM cellulose chromatography; the number in the index indicates the type of the parent collagen chain (1 or 2 in the case of collagen type I composed of two types of parent chains, 1 in the case of collagen type III which is composed of a single type of collagen parent chain only). Preparation of the nine standards used for spiking in different electrokinetic separations was also described previously [11]. The following peptides were used: $\alpha_1(I)CB_2$, $\alpha_1(I)CB_3$ (M_r 3300), $\alpha_1(I)CB_4$ (M_r 4600), $\alpha_1(III)CB_3$ (M_r 8600), $\alpha_1(I)CB_6$ (M_r 16 500), $\alpha_1(I)CB_7$ and $\alpha_1(I)CB_8$ (M_r 24 000), $\alpha_2(I)CB_4$ (M_r 29 000) and $\alpha_2(I)CB_{3,5}$ plus [$\alpha_1(III)CB_9$]₃ (both M_r about 60 000). The purity of these isolates was checked by routine polyacrylamide gel electrophoresis (PAGE) and reversed-phase chromatography as described in Refs. [14] and [15]. Samples for analysis were solubilized in 5% formic acid at a con-

centration 1.5 mg/ml. It is necessary to stress that the $\alpha_1(I)CB_3$ peptide can be released only after a prolonged CNBr cleavage time. If shorter times are used $\alpha_1(I)CB_2$ peptide is released only which in most separations runs closely to the former one. If fractions enriched in a particular peptide were needed the separations were repeated (about 20 times) and the fraction of interest was accumulated in the vial close to the detection window.

3. Results and discussion

If a sample of the CNBr-released peptides from rat tail tendons is run in 20 mmol/l phosphate buffer only a single, strongly fronting peak was observed at 12.5 min migration time (Fig. 1a). If the same buffer was made 35 mmol/l with respect to SDS no peak appeared at all. However if reversed polarity was applied the profile seen in Fig. 1b was obtained in which the slowest peak occurring at 11.6 min running time was identified as $\alpha_1(I)CB_6$. Applying a microemulsion system (35 mmol/l SDS in 20 mmol/l phosphate buffer containing 6.6 mass% of each *n*-butanol and hexane and keeping the apparent pH at 7.0) the profile seen in Fig. 1c was obtained. Increasing the pH to 8.0 yielded a similar profile though the individual peaks were a slightly better separated and the whole profile was slightly shifted to the cathodic side (inlet) of the capillary. This effect could be explained by an increase in the endosmotic flow which in this arrangement is directed to the right hand side of the electropherogram. If the pH was further increased (up to pH 9.0) additional spreading of the electropherogram and shifting to the right hand side of the recording of particularly the last two peak was observed (data not shown), however, no separation of the peptides contained in the bulky peak at the anodic side of the electropherogram occurred. The three peaks at the end of the electropherogram were identified by spiking as $\alpha_1(I)CB_8$, $\alpha_1(I)CB_7$ and $\alpha_1(I)CB_6$, respectively. Separation of the respective CNBr peptide standards (prepared by reversed-phase chromatography) is shown in Fig. 2, which confirms the identification. It is noticeable that under these conditions two peaks, namely $\alpha_1(I)CB_7$ and $\alpha_1(I)CB_8$

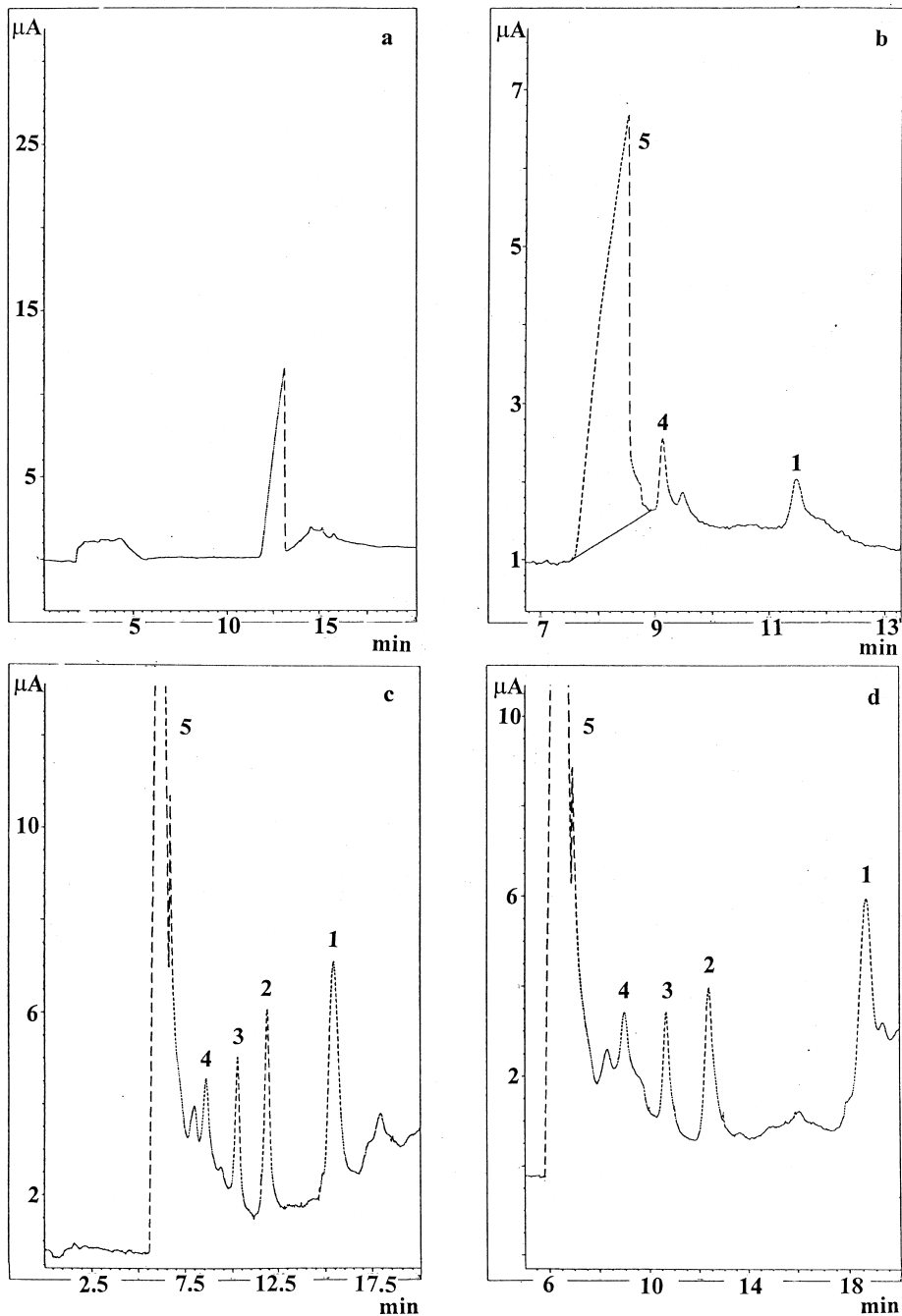


Fig. 1. Separation of CNBr-released peptides from rat tail tendon collagen: (a) 20 mmol/l phosphate buffer, 57 (50 cm to the detector) \times 75 μ m I.D. capillary, pH 7.0, detection by UV at 214 nm, positive polarity 10 kV per capillary; (b) as (a) but with a buffer of 35 mmol/l with respect to SDS, negative polarity; (c) as (a) but with a buffer of 35 mmol/l SDS and 6.6 mass% of each *n*-butanol and *n*-hexane added, negative polarity; (d) as (c) except that the apparent pH of the background electrolyte was increased to 8.0. Peak identification: 1= α_1 (I)CB₆ (16 500); 2= α_1 (I)CB₂ (24 000); 3= α_1 (I)CB₃ (24 000); 4=unidentified; 5=the bulk of all other peptides present in the mixture. Numbers in parentheses indicate the relative molecular mass of individual peptides.

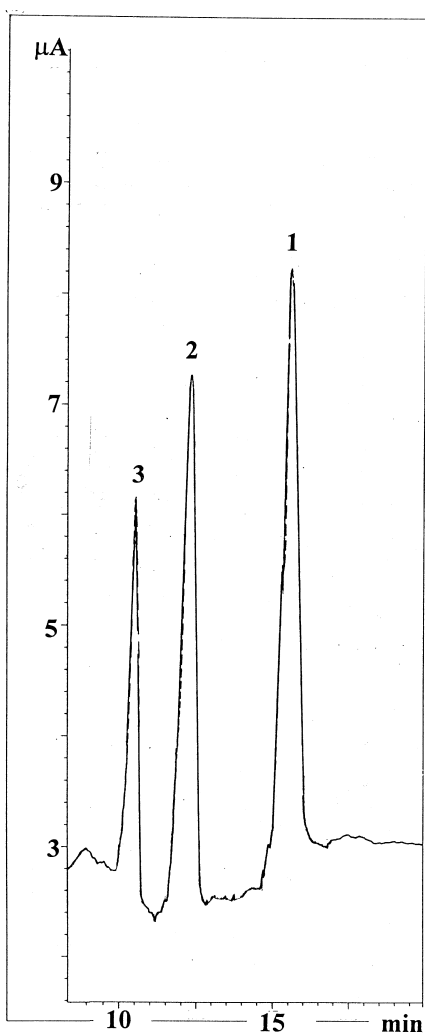


Fig. 2. Separation of a three-component standard CNBr peptide mixture in the same system as in Fig. 1c. Peak identification: 1= α_1 (I)CB₆; 2= α_1 (I)CB₇; 3= α_1 (I)CB₈; note that peptides 2 and 3 possess the same rel. mol. mass.

possessing identical M_r values of 240 000 were clearly separated. Next we tested a number of microemulsion systems using *n*-propanol, *n*-butanol, *n*-amyl alcohol as cosurfactants and *n*-hexane and cyclohexane as the emulsion forming solvents, however, no improvement of the separation was obtained.

In a separate set of experiments we attempted to decrease the polarity of the background electrolyte used. The idea was that if an organic solvent is

added to the micellar system, the partition conditions must be altered to create the possibility to improve the selectivity for a set of compounds separated on the basis of their hydrophobic properties. For this purpose 25 mmol/l borate buffer (50 mmol/l with respect to SDS), pH 8.0 containing 20% methanol was used. The result seen in Fig. 3 was obtained. Though no baseline separations were obtained in

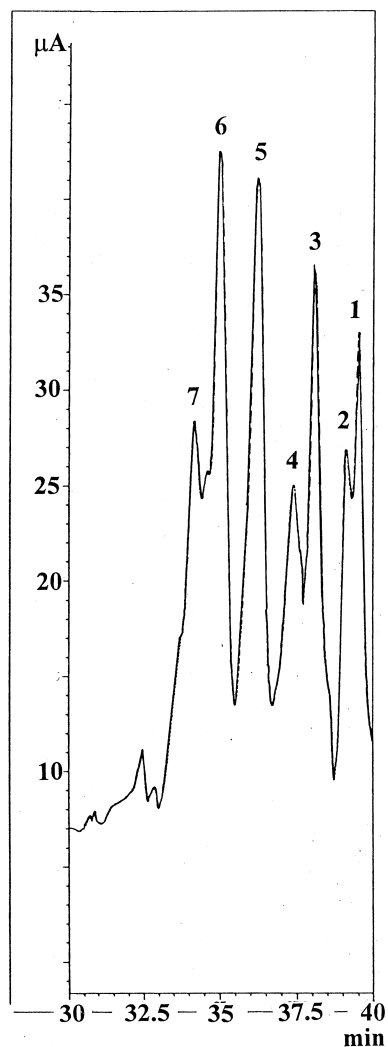


Fig. 3. Separation of the CNBr peptide mixture in the same capillary as above (Fig. 1) in 25 mmol/l borate buffer containing 50 mmol/l SDS and 20% methanol, 10 kV per capillary, negative polarity mode. Peak identification: 1= α_1 (I)CB₆; 2= α_1 (I)CB₇; 3= α_1 (I)CB₈; 4=unidentified; 5= α_2 (I)CB₄; 6= α_2 (I)CB_{3,5}; 7=[α_1 (III)CB₉]₃.

most cases, the position of $\alpha_1(\text{I})\text{CB}_6$, $\alpha_1(\text{I})\text{CB}_7$, $\alpha_1(\text{I})\text{CB}_8$, $\alpha_2(\text{I})\text{CB}_4$, $\alpha_2(\text{I})\text{CB}_{3,5}$ and a shoulder of $[\alpha_1(\text{III})\text{CB}_9]_3$ could be clearly traced in the profile. We were not able to determine the position of the remaining peptides which should be theoretically present in the mixture. This may be the reason for the occurrence of an unidentified peak occurring between $\alpha_2(\text{I})\text{CB}_4$ and $\alpha_1(\text{I})\text{CB}_8$. Though this separation is far from being capable to separate all the components present in the mixture it is still comparable to other separation techniques used for this purpose, e.g., slab gel electrophoresis, though the latter is worse and exhibits different selectivity in comparison to the capillary electrophoresis in acid media as reported previously [11]. Also we cannot claim that the peaks seen on the electropherogram represent pure peptides. If methanol was replaced by ethanol at the same concentration the profile appeared sooner before the detector's window owing to the smaller endosmotic flow: $\alpha_1(\text{I})\text{CB}_6$, $\alpha_1(\text{I})\text{CB}_7$ with a shoulder of $\alpha_1(\text{I})\text{CB}_8$, $\alpha_1(\text{I})\text{CB}_4$, $\alpha_1(\text{I})\text{CB}_2$, $\alpha_2(\text{I})\text{CB}_4$, $\alpha_1(\text{I})\text{CB}_{3,5}$ with a partly resolved peak of $[\alpha_1(\text{III})\text{CB}_9]_3$ could be clearly identified (Fig. 4). Similarly to the previous methanol containing system a peak which could not be ascribed to any of the marker (standard) peptides occurred between $\alpha_1(\text{I})\text{CB}_2$ and $\alpha_2(\text{I})\text{CB}_4$. The shape of the peak indicated that it may be composed of more than a single peptide. A serious drawback of all systems involving surfactants (micelles or microemulsions) described so far is that particularly the small peptides are lost in the profile and apparently comigrate with the larger ones as owing to small (if any) hydrophobic domains they do not associate easily, if at all, with the surfactant formed micelles/emulsions. Another problem is the presence of incompletely split peptides, as demonstrated by the presence of the $\alpha_2(\text{I})\text{CB}_{3,5}$ peptide.

The present results support further our previous suggestion that in the separation of collagen released CNBr peptides hydrophobic interactions play a role. This proposal was based on the similarity of the profiles obtained by reversed-phase chromatography and capillary electrophoresis in the acid media (pH 2.5) and by the observation that by hydrophobic modification of the capillary the separation effect is lost as probably most of the peptides adhere strongly to the hydrophobic inner surface of the capillary

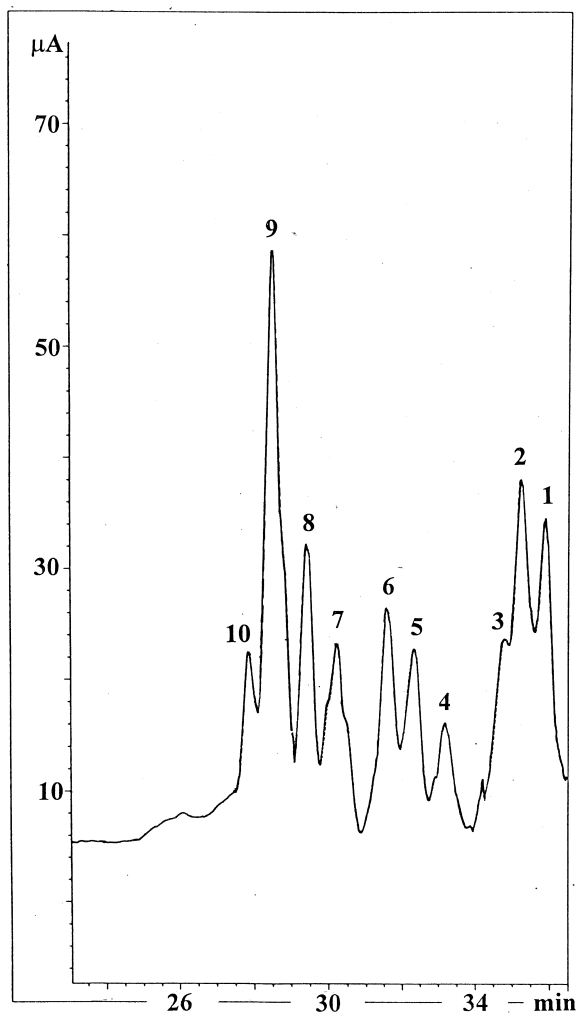


Fig. 4. Separation of the CNBr peptide mixture under the same conditions as in Fig. 3, except that 20% methanol was replaced by the same proportion (i.e., 20%) ethanol. Peak identification: 1 = $\alpha_1(\text{I})\text{CB}_6$; 2 = $\alpha_1(\text{I})\text{CB}_7$; 3 = $\alpha_1(\text{I})\text{CB}_8$; 4 = $\alpha_1(\text{III})\text{CB}_4$; 5 = $\alpha_1(\text{I})\text{CB}_4$; 6 = $\alpha_1(\text{I})\text{CB}_2$; 7 = unidentified; 8 = $\alpha_2(\text{I})\text{CB}_4$; 9 = $\alpha_2(\text{I})\text{CB}_{3,5}$; 10 = $[\alpha_1(\text{III})\text{CB}_9]_3$.

[10,11]. In this communication we bring another fact supporting this idea, namely in the microemulsion system two peptides of identical molecular mass, but different in their lipophilicity can be clearly separated.

Though the large (most hydrophobic) members of the set exhibited (due to their association with the strongly negatively charged surfactant) a strong anodic mobility in the microemulsion system, the

separation window for the smaller peptides was too short to allow sufficient time for a good separation. If the polarity of the background electrolyte was decreased by adding 20% of methanol or ethanol to the micellar system, more peaks (better selectivity) was obtained and the position of a number of peaks could be identified in the profile, still the conditions were not sufficient to separate all the components of the mixture.

At this stage it appeared very likely that with different modifications of the micellar microemulsion system good separation of all the individual members

of this complex mixture was unlikely to be achieved. There were, however, three other challenging arrangements to be investigated, namely separation in a background electrolyte capable of molecular sieving (both in presence and absence of SDS) and separation in a reversed-phase packed capillary. The results are seen in Figs. 5–7. With 10% polyethylene oxide added to the background electrolyte we were able to discern 27 peaks of the 24 theoretically present peptides, though, admittedly, no baseline separation was seen of most of them. (It appears likely that the excessive three peaks seen in the

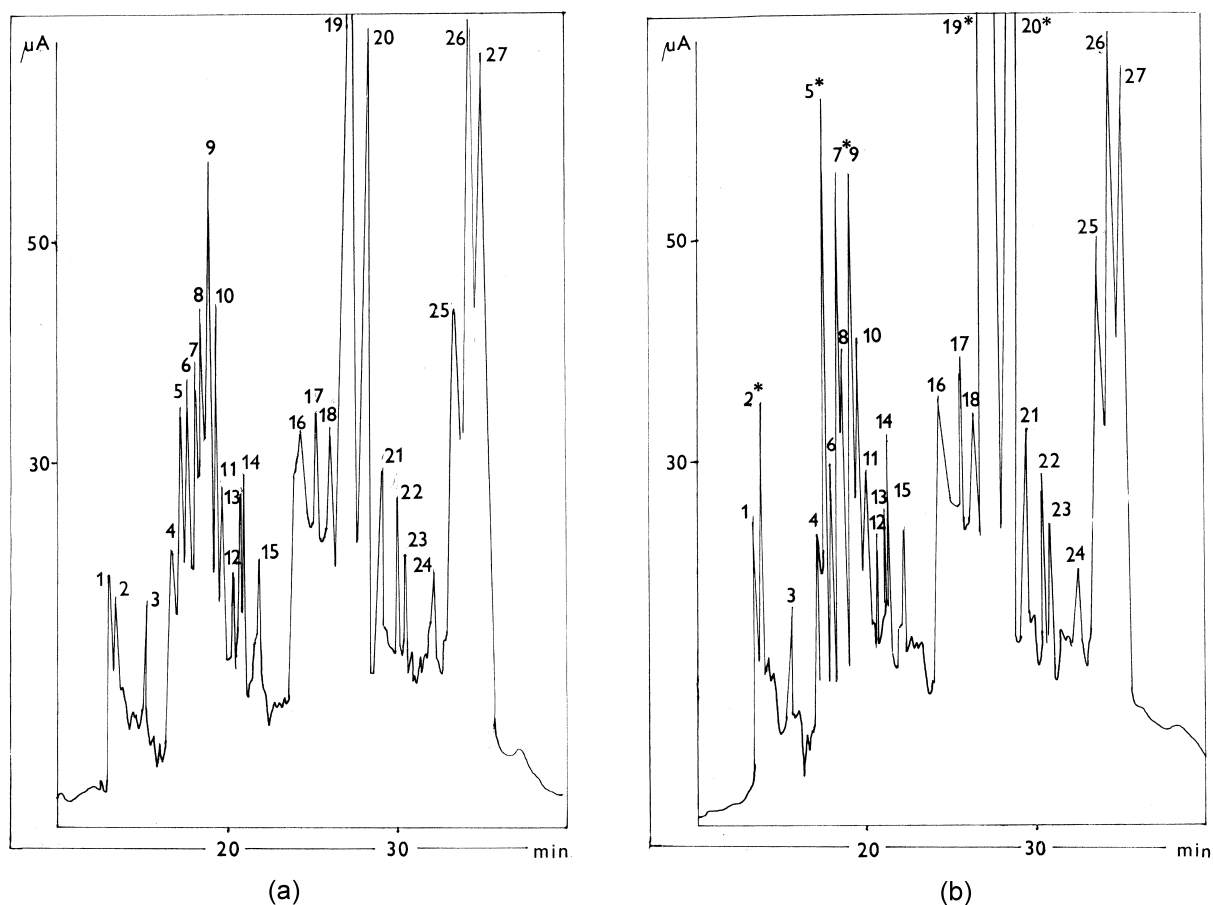


Fig. 5. (A) Separation of the CNBr-released peptide mixture in 20 mmol/l phosphate buffer, pH 8.5 containing 10% polyethylene oxide at 15 kV per capillary, positive polarity mode. Peak identification: 1= $\alpha_1(\text{I})\text{CB}_2$; 2= $\alpha_1(\text{I})\text{CB}_3$; 3=unidentified; 4= $\alpha_1(\text{I})\text{CB}_4$; 5= $\alpha_1(\text{III})\text{CB}_3$; 6= $\alpha_1(\text{III})\text{CB}_6$; 7= $\alpha_1(\text{III})\text{CB}_4$; 8= $\alpha_1(\text{I})\text{CB}_5$; 9= $\alpha_1(\text{I})\text{CB}_6$; 10–15=unidentified; 16= $\alpha_1(\text{I})\text{CB}_6$ (containing the non-helical region); 17=unidentified; 18= $\alpha_1(\text{III})\text{CB}_5$; 19= $\alpha_1(\text{I})\text{CB}_7$; 20= $\alpha_1(\text{I})\text{CB}_8$; 21–24=unidentified; 25= $\alpha_1(\text{I})\text{CB}_4$; 26= $\alpha_2(\text{I})\text{CB}_{3,5}$; 27= $[\alpha_1(\text{III})\text{CB}_9]_3$. (B) Spiked profile. Conditions as in (A). The same mixture was spiked by peptide fragments Nos. 1, 2, 5, 7, 19 and 20 (marked by asterisk) that were homogenous in PAGE (see Experimental). The identity of remaining peaks was determined by spiking the electrophoregram with fractions which were enriched in the particular peak only, however contaminating zones could be seen in PAGE.

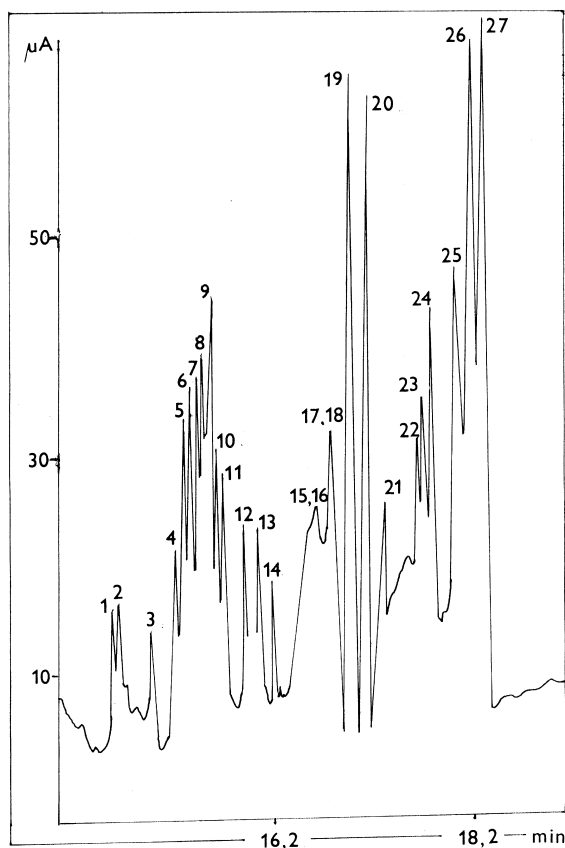


Fig. 6. Separation of CNBr-released peptides in the same buffer as in Fig. 5 except that the background electrolyte was made 35 mmol/l with respect to SDS. Reversed polarity mode, peak identification as in Fig. 5A.

electropherogram may result from incomplete cleavage of the structure at methionine residues or may represent contaminants from the tissue sample). In this case the separation was run in the absence of SDS in 20 mmol/l phosphate buffer, pH 8.5, using a positive polarity mode (anode at the inlet of the capillary, run voltage 15 kV). In the overall peptide profile (Fig. 5a), peaks Nos. 1, 2, 5, 7, 19 and 20 were identified by spiking with the respective peptide isolates (Fig. 5b). The identification of the other peaks in the profile offered similar results (data not shown).

If the same buffer was made 35 mmol/l with respect to SDS, (and 10% of polyethylene oxide was added) no peaks were observed in the positive mode; however with reversed polarity the separation seen in

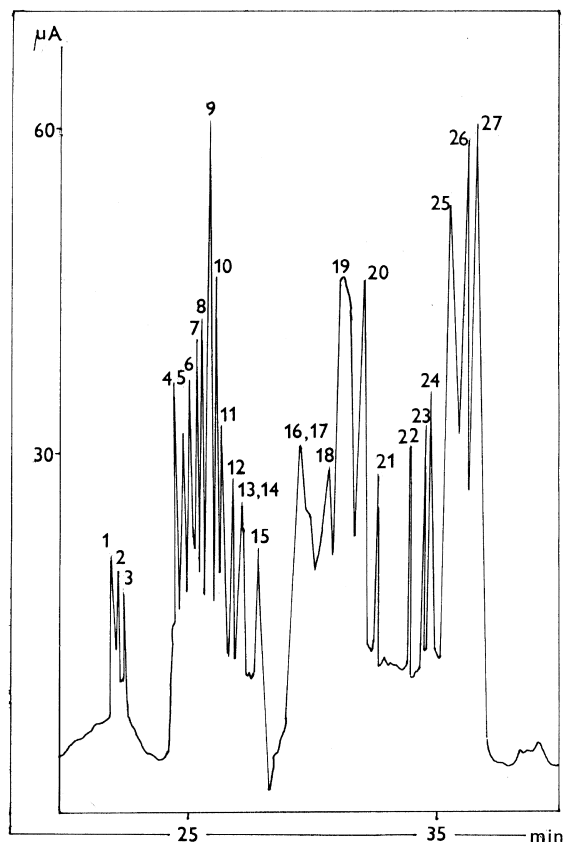


Fig. 7. Electrokinetically-driven separation of CNBr-released tendon collagen peptides on a column packed with 3 μm Hypersil C_8 . Buffer composition: 3 ml acetonitrile, 2 ml 40 mmol/l triethylamine-phosphoric acid, pH 3.0, water added to 10 ml total volume. 15 kV per capillary, positive polarity mode. For peak identification see Fig. 5A.

Fig. 6 was obtained: the main peptides were separated according to their relative molecular mass. This result could have been predicted keeping in mind the results that are routinely obtained with slab gel electrophoresis. Note that the sequence of individual peptides is just reversed when compared to the previous separation in the absence of SDS.

Table 1 summarizes the reproducibility of migration times and relative peak areas for the five peptides the position of which was identified by spiking (see Fig. 5).

Another good separation was obtained with a Hypersil C_8 (3 μm) packed column. The run buffer (mobile phase) was composed of 3 ml acetonitrile, 2

Table 1

Reproducibility of the migration times and peak area of selected peptides in the CNBr peptide profiles ($n=20$) during the micellar electrokinetic separations with and without SDS

Peak No.	Peptide	Reproducibility of the migration times			Reproducibility of the relative peak area (intra-assay precision), RSD (%)
		Mean (min)	SD (min)	RSD (%)	
<i>(A) Without SDS^a</i>					
2	$\alpha_1(\text{I})\text{CB}_3$	13.55	0.38	2.80	7.93
5	$\alpha_1(\text{III})\text{CB}_3$	17.68	0.53	3.00	8.55
7	$\alpha_1(\text{III})\text{CB}_4$	18.31	0.67	3.66	9.47
19	$\alpha_1(\text{I})\text{CB}_7$	26.92	0.70	2.60	9.86
20	$\alpha_1(\text{I})\text{CB}_8$	28.34	0.87	3.07	10.23
<i>(B) With SDS^b</i>					
2	$\alpha_1(\text{I})\text{CB}_3$	14.72	0.15	1.02	8.01
5	$\alpha_1(\text{III})\text{CB}_3$	15.36	0.17	1.11	9.02
7	$\alpha_1(\text{III})\text{CB}_4$	15.49	0.25	1.61	9.35
19	$\alpha_1(\text{I})\text{CB}_7$	16.98	0.37	2.18	9.37
20	$\alpha_1(\text{I})\text{CB}_8$	17.25	0.59	3.42	11.26

^a (A) Separation in 20 mmol/l phosphate buffer, pH 8.5 containing 10% polyethylene oxide at 15 kV, positive polarity mode.

^b (B) Separation under the same conditions as (A) except that the background electrolyte was made 35 mmol/l with respect to SDS (reversed polarity).

ml 40 mmol/l triethylamine–phosphoric acid buffer (TEAP) of pH 3.0 and water added to the total volume of 10 ml. Separation was run at 15 kV per capillary. As seen in Fig. 7, 25 rather clearly separated peaks were observed of which 17 were identified. In this case it is feasible to assume that the separation is based mainly on the hydrophobic properties of the solutes and the stationary phase. The fact that one additional peak than the expected 24 was found can be ascribed to the incomplete cleavage at some of the methionine residues (for instance a part of the $\alpha_2(\text{III})\text{CB}_{3,5}$ can be cleaved into the constituting peptides increasing thus the total number of peptide peaks by two).

Comigration of peak Nos. 13+14 and 16+17 seen in the profile shown in Fig. 6 and peak Nos. 15+16 and 17+18 in the profile in Fig. 7 was ascertained by spiking the sample by fractions enriched in peptides Nos. 14, 16 and 18.

4. Conclusions

The following conclusions can be drawn from the above described results:

1. The separation of collagen type I and III CNBr-released peptides can be carried out in two different separation modes in which either the molecular sieving or hydrophobic partition play the dominating role.
2. In electrokinetically-driven separations (unpacked capillaries) the best (most complete) separations can be obtained either in the presence of polyethylene oxide in the background electrolyte (10%) at 15 kV per capillary (runs effected at pH 8.5 in the positive mode), or in the presence of 20 mmol/l SDS and 10% polyethylene oxide under the same conditions, however with reversed polarity.
3. Micellar and microemulsion chromatography offer selective separation of the $\alpha_1(\text{I})\text{CB}_6$ peptide (micellar system) or $\alpha_1(\text{I})\text{CB}_6$, $\alpha_1(\text{I})\text{CB}_7$ and $\alpha_1(\text{I})\text{CB}_8$ peptides (microemulsion system). However in these cases separation must be carried out in the reversed polarity system.
4. Addition of polyethylene oxide (10%) to the background electrolyte at pH 8.5 allows the separation of 27 peptides of which the positions of 17 were identified by spiking. This result indicates that incomplete cleavage at some methionine residues may have occurred (the

number of peaks observed was higher than theoretically expected). If SDS is added a very similar result is obtained, however, on the contrary to the former arrangement this separation must be run in the reversed polarity mode.

5. Another possibility yielding 25 peptide peaks out of the 24 theoretically possible was obtained by electrodriven chromatography (no SDS present) using a 3 μm Hypersil C₈ packed capillary with acetonitrile as organic modifier and TEAP buffer at pH 3.

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