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# Micropreparation of tissue collagenase fragments of type I collagen in the form of surfactant–peptide complexes and their identification by capillary electrophoresis and partial sequencing

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## Abstract

Combination of standard approaches like pepsin digestion and slab gel electrophoresis with capillary separations allows a relatively easy identification of in vivo occurring collagen fragments. Capillary electrophoresis can be done either in 25 mM phosphate buffer (pH 2.5) or in a 25 mM phosphate buffer (pH 4.5) made 0.1% with respect to sodium dodecyl sulfate (SDS). While in the first case peptides move to the cathode in a molecular mass dependent manner, in the second case they move towards anode (also in a molecular mass dependent manner). The profiles obtained by the two approaches resemble mirror images with low molecular mass peptides moving first in the acid background electrolyte while they move last in the presence of SDS. It is proposed that in the capillary electrophoretic separation at pH 2.5 the separation mechanism involves the interaction of the individual peptides with the capillary wall while in the second case (pH 4.5) the leading mechanism of separation involves the interaction of the analytes with the micellar phase. For micellar phase separation the system must be run at reversed polarity. Capillary electrophoretic separation in the pH 2.5 buffer is considerably affected by the presence of SDS in the previous steps of peptide preparation. If the peptides are obtained from SDS slab gel electrophoresis, their movement in the capillary electrophoresis step is about three times faster than the movement of corresponding peptides which have not been complexed with SDS. © 1998 Published by Elsevier Science B.V.

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## 1. Introduction

In studying protein metabolism under the action of exogenous noxae, complex separation procedures must be applied to reach the desired goal. It is advantageous to combine standard approaches (like slab gel electrophoresis of pepsin extracted insoluble collagens) with more recent techniques, in our case capillary electrophoresis (CE).

Following the success of separating most diverse

solutes by micellar electrokinetic chromatography, a number of authors attempted to exploit the hydrophobic properties of proteins for their separation by this electrokinetically driven technique ([1,2], for review see [3]). Micellar electrokinetic protein separations are based on the fact that the mobility of a charged micelles is greater than that of any of the proteins and protein–micelle complexes and that, reflecting the hydrophobic properties of protein polypeptide chains, the equilibria between micelles and solutes will differ because of the differences in amino acids constituting a particular polypeptide

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chain and their sequence. One of the first successful attempts in this respect was published by Strege and Lagu [4] who were capable of separating successfully a model mixture composed of lysozyme, ribonuclease, myoglobin,  $\beta$ -lactoglobulin and bovine serum albumin under a variety of pH and surfactant conditions. Both cationic and anionic surfactants [e.g. cetyltrimethylammonium bromide or sodium dodecyl sulfate (SDS)] were investigated in this respect.

Protein migration times were shown to increase as surfactant concentration increased. The concentration of surfactants used should exceed the critical micelle concentration in order to ensure complete saturation of the capillary wall and minimize protein–wall interactions [3]. Also additional organic modifiers, like acetonitrile were successfully applied to increase further the selectivity of micellar systems for small peptides [5].

Experimental evidence has been provided for different mechanisms involved in separating proteins in the presence of surface active agents. Thus with cetyltrimethylammonium chloride (0.1% in the background electrolyte) protein–micelle, protein–surfactant monomer and protein–surfactant monomer–micelle equilibria can be apparently involved. As indicated already by using surface active agents in the background electrolyte, the inner capillary surface can also be modified (typically with  $C_{18}$  modified capillaries); the sorption of the surface active agent results in a strong, pH independent endosmotic flow and superior injection-to-injection time reproducibility. However to ensure such phenomena to occur, a sufficient amount of the surface active modifier (beyond the critical micelle concentration [3]) must be available in the background electrolyte.

A review summarizing the current knowledge about micellar electrokinetic separation of proteins has been prepared by Strege and Lagu [3]. A wide variety of proteins have been investigated. Erichsen and Holm [6] reported separation of a serine protease (savinase), Yashima et al. [5] used micellar systems for separating closely related motilin peptides; a lot of attention has been paid to separation of glycoproteins (antibodies) and the clinical potential of this separation technique (Alexander and Hughes [7], Tadey and Purdy [8]). It is, perhaps, not surprising that considerable success was achieved in separating lipoproteins owing to their high affinity for lipophilic

compounds. A study on micellar electrokinetic chromatography of a series of peptides (8–31 amino acids) led to the conclusion that for separating peptides containing more than 20 amino acids organic modifiers are necessary to add to the micelle containing background electrolyte, assuming that the hydrophobicity of peptides increases with increasing size [9].

In a previous paper [10] we have demonstrated that separation of CNBr peptides derived from collagen type I in acid buffers (pH 2.5) reflects their molecular size, larger peptides being eluted later than the smaller ones. It was hypothesized that the separation reflects the number of domains capable of interaction with the bare silica capillary wall; owing to the large internal homogeneity of collagen polypeptide chains, larger peptides must necessarily contain more interactive loci than the shorter ones because the overall profile obtained by CE was practically the same as the profile obtained by reversed-phase chromatography; a linear relationship between migration time and molecular mass of separated collagen peptides in the overall profile was observed no matter whether CE at low pH (2.5) or reversed-phase chromatography were used.

In preliminary tests with collagen derived peptides we have found that their interaction with SDS is very strong and that the surfactant is practically impossible to remove from collagen polypeptide chains after extensive washing with buffers of different pH, even if the wash buffers contained an organic modifier (acetonitrile) or another surface active agent (Triton X-100) (unpublished).

This stimulated our efforts to separate collagen polypeptide chain fragments in the form of surfactant containing complexes. It seemed feasible to assume that under appropriate conditions the protein–capillary wall interactions will be weakened, thus preventing peak tailing. On the other hand it was also assumed that collagen–micelle interactions will exhibit sufficient affinity differences with different collagen fragments to yield good selectivity for the otherwise difficult to separate collagen constituting peptides.

We found recently that exposure of rats to chronic hypoxia results in the presence of collagen fragments in the peripheral pulmonary arteries [11]. These in vivo occurring collagen cleavages were compared

with the collagen fragments prepared in vitro by tissue collagenase from collagen type I.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals used were either of a p.a. grade or highest available purity; collagen type I (from the rat tail), acrylamide, ammonium persulfate, EDTA, SDS, N,N,N',N'-tetramethylethylenediamine (TEMED), mercaptoethanol, glycine, Tris base, Coomassie Brilliant Blue R, Triton X-100 and 4-chloro-1-naphthol were obtained from Sigma (Sigma-Aldrich, Deisenhofen, Germany), SDS-PAGE molecular mass standards, low range from Bio-Rad (Bio-Rad, Hercules, CA, USA), acid soluble collagen type I (ASC) from the calf skin and sheep antibodies to collagen type I were a gift of Professor Adam prepared in the Rheumatology Institute (Prague) and HRP-conjugated immunoglobulins against sheep and goat immunoglobulins (Su ASG/Px) were obtained from USOL (Prague, Czech Republic). Milli Q Water (Millipore, Watford, USA) was used for preparing the CE buffers and for rinsing the capillary. All other chemicals needed for the preparation of the background electrolyte were obtained from Lachema, Brno, Czech Republic.

### 2.2. Capillary electrophoresis

Capillary electrophoresis of peptide fragments was done with Beckman PACE 5500 (Fullerton, CA, USA) using fused-silica capillary 57 cm (50 cm to the detector) × 75 μm capillary run at 15 kV and 25 mM phosphate buffer in pH 2.5 or alternatively in pH 4.5 buffer containing 0.1% SDS as background electrolyte. Between runs the capillary was washed with Milli-Q water (4 min), 1 M NaOH (6 min), Milli-Q water (4 min), 1 M HCl (8 min) and finally with Milli-Q water again (4 min). Before each run the capillary was equilibrated with the background electrolyte (2 min). Samples were injected by overpressure (3.5 kPa, 1 s).

### 2.3. Model system: cleavage of collagen type I with tissue collagenase

Incubation with tissue collagenase was done according to the procedure described by Krane et al. [12]. Briefly, 5 mg of commercial collagen type I preparation was incubated at 20°C overnight in 50 mM Tris HCl buffer (pH 7.5) made 150 mM with respect to NaCl and 10 mM with respect to CaCl<sub>2</sub> and 1 μM ZnSO<sub>4</sub> (substrate:enzyme ratio 1:50) and the reaction was stopped by the addition of EDTA to a final concentration 50 mM after 12 h; the samples were stored at -5°C until used for further analysis by capillary or polyacrylamide gel electrophoresis.

### 2.4. Biological system: preparation of collagen from lung arteries

#### 2.4.1. Preparation of arteries

Five rats (Wistar strain) of average body mass (b.w.) were exposed to chronic isobaric hypoxia (Fi<sub>O<sub>2</sub></sub> = 0.1, 3 weeks [13]) and anesthetized with pentobarbital (100 mg/kg b.w., i.p.). After thoracotomy and heparinization, the pulmonary artery and the left heart ventricle were cannulated. The lungs were perfused at 0.06 ml/min/g b.w. with about 70 ml of cold physiological salt solution containing 4% (w/v) albumin. The lungs were then excised and the third to fifth branches of pulmonary artery (peripheral pulmonary artery, PPA) were isolated under a dissecting microscope; 14–21 vessels from the left and the right lungs ranging in diameter from 100–400 μm were dissected in each animal. The length and diameter in situ of each peripheral pulmonary artery were measured by eyepiece micrometer and the samples were weighed (wet mass). All samples were carefully stripped of surrounding connective tissue. Segments of the vessels were cut into small pieces, washed in distilled water and lyophilized. Fragments of parent collagen type I α-chains were obtained from animals subjected to chronic hypoxia (see [11] for the experimental procedure).

#### 2.4.2. Collagen preparation from arteries

Noncollagenous proteins were removed from samples after incubation in 15 volumes of 4 M

guanidine-HCl in 0.05 M (CH<sub>3</sub>COO)<sub>2</sub>Na buffer (pH 5.8), 48 h in 4°C. After washing in distilled water, the remaining tissue was pepsinized by 10 volumes of 2% (w/v) of pepsin in 0.5 M CH<sub>3</sub>COOH (pH 2.5), 4 h at room temperature and 20 h at 4°C, centrifuged (8000 g, 30 min), and the supernatant was lyophilized.

## 2.5. Additional procedures

### 2.5.1. Gel electrophoresis

Gel electrophoresis separations [SDS-polyacrylamide gel electrophoresis (SDS-PAGE)] were performed by method of Laemmli [14] on discontinuous slab gel using 4% stacking gel and 7.5% separating gel. The electrophoretic separation was run in Tris-glycine buffer system (pH 8.3) with and without reduction. The gels were stained for 1 h with 0.25% Coomassie Brilliant Blue R in methanol-acetic acid-water (40:10:50, v/v/v). Destaining was performed for 1 h with methanol-acetic acid-water (40:10:50, v/v/v).

### 2.5.2. Immunoblotting detection of collagenous proteins

To prove the collagenous nature of the fragments found in lung arteries the proteins after slab gel electrophoresis were transferred to the nitrocellulose membrane in 15 mM sodium borate buffer, pH 9.2, 24 h at 4°C. Starting transfer power conditions were 25 V/250 mA, finishing conditions were 25 V/350 mA. Sheep anticollagen type I antibodies were diluted 1:20 in 2% skimmed milk-PBS and nitrocellulose membrane was incubated in this solution 1 h at room temperature, washed with 2% skimmed milk-PBS. Membrane was then incubated in a horseradish peroxidase (HRP)-conjugated antisheep-goat antibodies solution diluted 1:500, 1 h at room temperature. After washing, the membrane was stained with HRP substrate, 4-chloro-1-naphthol (15 mg in 5 ml of methanol, 20 ml 10 mM Tris-HCl, 0.04% H<sub>2</sub>O<sub>2</sub>). The reaction was allowed to proceed in the dark for 15 min until all bands were visualized. The membrane was then air dried. This procedure was used to prove the identity of collagen type I fragments released from the lung samples.

### 2.5.3. Aminoterminal peptide sequencing

Collagenase treated commercial collagen preparations or lung collagen isolates were separated by CE and individual peptides were accumulated in separate cathodic vials which were interchanged at appropriate time intervals (calculated on the basis of migration velocity and the length of the capillary between the detection window and the end of the capillary) after stopping, exchanging the electrode vial and restarting the electrophoretic run. In this way material from fifty automated runs was collected. The solution in cathodic vials was lyophilized and subjected to aminoterminal sequencing as described in [15,16].

### 2.5.4. Separated fragments recovery from the polyacrylamide gel

Zones revealed by the staining procedure were cut out with a blade, the gel pieces were placed into a microcentrifuge tube, the dye was removed by adding 1 ml of wash buffer and sonicating for 5–15 min at 60°C until clear gels. The wash buffer was removed, 50–100 µl of the extraction buffer (100 mM NaHCO<sub>3</sub>, 8 M urea, 3% SDS, 0.5% Triton X-150, 25 mM dithiothreitol) was added and incubated 20–30 min at 65°C. The gel was then homogenized with an Eppendorf fitting pestle VWR Cat. No. KT 749515-0000 from Amicon (Beverly, MA, USA) and the tube with homogenized gel was incubated at 50–60°C overnight. Next 100 µl of the extraction buffer (see above) was added and the gel slurry was transferred into an Amicon Microcon inserted with Micropore inset. The tube was rinsed with 100 µl of the extract buffer and this rinse was also transferred into the Micropore inset. Next the assembly was spun until all liquid was removed from the Micropore inset (13 000 g, 20–30 min). Collagen fragments were retained above the Microcon membrane; the sample was transferred to a new vial and lyophilized. For CE the lyophilizates were dissolved in 500 µl of the pH 2.5 or pH 4.5 background electrolyte buffer (the latter containing 0.1% SDS).

### 2.5.5. Preparation of molecular mass standards

Calibration of the CE was done with cyanogen bromide peptides of collagen type I prepared as described in our previous communication [10]. Prep-

aration of surfactant–peptide complexes was done as described above.

### 3. Results

Polyacrylamide slab gel electrophoresis of the collagen preparations from hypoxia affected pulmonary vessels revealed the profile shown in Fig. 1. Aside to collagen  $\alpha$ -chains, their dimers and higher polymers, two distinct bands were detected in the front of the electropherogram corresponding to polypeptides of 66 and 45.10<sup>3</sup> rel. molecular mass units, provided that the standard set of globular proteins was used for calibration (preparations from animals which were not subjected to hypoxia were devoid of these fragments). When the whole front section of the electropherogram (below the  $\alpha$ -chain region up to

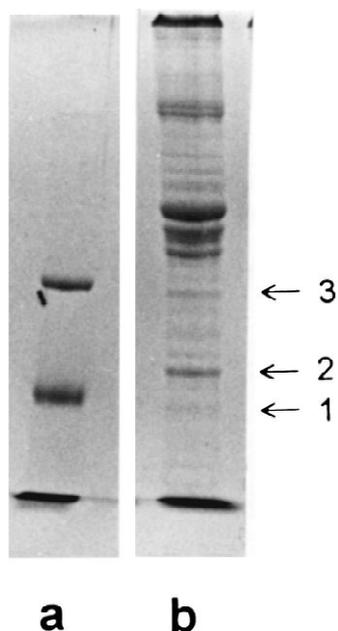


Fig. 1. Polyacrylamide slab gel electrophoresis of the collagen fraction released from lung vessels. Discontinuous gel, 4% stacking gel, 7.5% separating gel; Tris–glycine buffer (pH 8.3) containing 0.1% SDS was used; staining and destaining by Coomassie Brilliant Blue as specified in Section 2. (a) molecular weights standards (66 000 and 45 000 rel. mol. mass); (b) collagen fractions from the walls of peripheral pulmonary arteries, from top to bottom:  $\beta$  fraction,  $\alpha_1$  chains (mixture of collagens I and III),  $\alpha_2$  chain (collagen I) and dominant low-molecular-mass peptides nos. 3, 2 and 1.

the front) was excised, homogenized and the polypeptide fraction isolated according to the procedure described in Section 2, CE in bare silica capillary run in 25 mM phosphate buffer (pH 2.5) revealed the profile shown in Fig. 2A. The separation was rather fast compared to fragments of similar size obtained after CNBr cleavage of a collagen sample (see [10]). It was assumed that the change in migration time may have been caused by the fact that in the preceding step (slab gel electrophoresis) the respective fractions interacted with SDS, and this remained when the peptidic moieties were extracted from the gel while the CNBr series of peptides had never been brought into the contact with SDS. Consequently a separation based on the hydrophobicity of the polypeptide–SDS complex resulting from slab gel electrophoresis appeared likely. When the sample was run in a 25 mM phosphate buffer pH 4.5 containing 0.1% SDS the profile shown in Fig. 2B was obtained. Note that the polarity of the run was reversed and no peaks were revealed when anode was used as the sample side and cathode was joined to the detector end of the capillary. The runs shown in Fig. 1 have the relation of a subject and its mirror image. When the zones separated by slab gel electrophoresis were isolated separately, it was possible to prove that the peak designed in Fig. 1 as no. 3 refers to a larger polypeptide while the peak no. 2 represents an entity of smaller rel. mol. mass. Peak no. 1 was a peptidic contamination that has not been further characterized. In Fig. 2A the peaks move practically under the action of their positive charge only as the endosmotic flow is very small (we were unable to detect the endosmotic flow marker even after 180 min of run time). In a previous paper [10] we have proposed that in the very acidic background electrolyte used, separation of type I collagen CNBr peptides is effected mainly through their interaction with the capillary wall. It was also demonstrated that the CNBr peptides are eluted in a sequence which reflects their molecular mass and that the migration time vs. molecular mass relationship is linear. Because collagen fragments occurring in hypoxia affected tissue fall within the range of molecular masses of CNBr released peptides they would be expected to move more slowly than observed owing to their expected molecular mass. Here it is necessary to emphasize the high internal homogeneity of

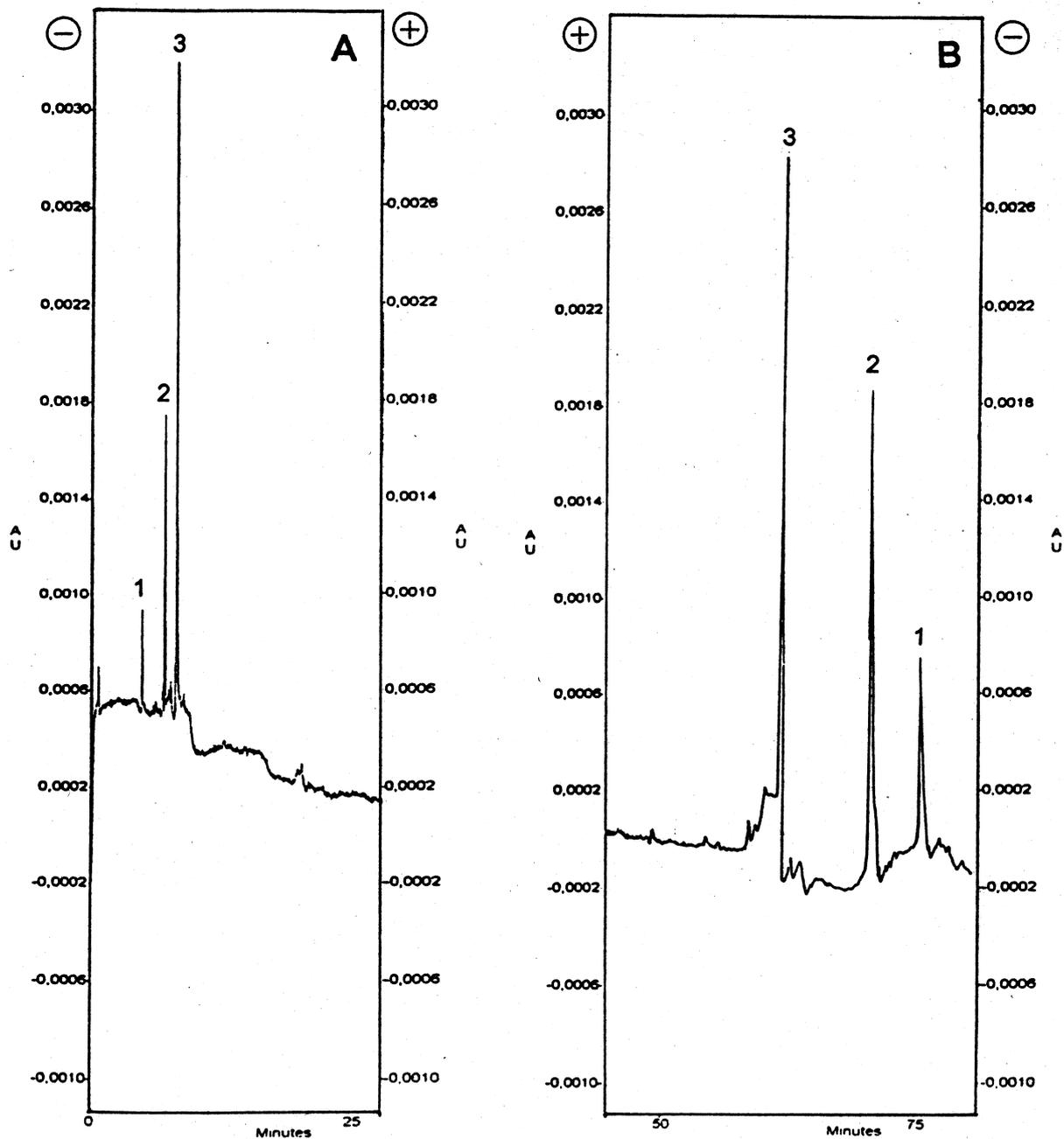


Fig. 2. Separation of the low molecular fraction (containing fragments up to  $66$  and  $45 \cdot 10^3$  rel. mol. mass according to the calibration with a standard set of proteins) by CE. Samples isolated from polyacrylamide slab gel run in SDS. (A)  $25$  mM phosphate buffer (pH  $2.5$ ),  $15$  kV; (B)  $25$  mM phosphate buffer (pH  $4.5$ ) containing  $0.1\%$  SDS,  $15$  kV. Polarity as indicated (A), normal polarity mode, (B), reversed polarity mode). Other conditions as specified in Section 2. Peak identification: 1,  $17 \cdot 10^3$  rel. mol. mass fragment; 2,  $34 \cdot 10^3$  rel. mol. mass fragment; 3,  $64 \cdot 10^3$  rel. mol. mass fragment. Calibration for mol. mass estimation by CNBr peptide fragments. Benzylalcohol used as endosmotic flow (EOF) marker. In (A) EOF marker was not seen before  $180$  min run time.

the collagenous sequences which justifies the above considerations. If the pulmonary vessel wall isolated polypeptides were still in the form of the SDS complexes, then it would not be surprising that their electrophoretic behaviour would be also different. Consequently it was worth while to attempt separation based on the presence of the surfactant molecules attached to the polypeptide moiety. Indeed when the separation was carried out in the micellar electrokinetic chromatographic mode (0.1% SDS) at pH 4.5, the profile shown in Fig. 2B was obtained. As expected the largest peptide, which can be bonded to more SDS molecules, moves faster than the smaller polypeptide which is capable of binding less SDS molecules. The molecules of the surfactant obviously enrich the separated peptides in their negative charge and, thus, the larger polypeptide is more enriched than the small one which results in a higher anodic mobility of the former. On the other hand the hydrophobic domain of the attached surfactant molecules makes the SDS–collagen polypeptides complexes prone to an interaction with SDS micelles present in the background electrolyte.

The above considerations were verified by using collagen-released CNBr peptides. As shown in Fig. 3A in the pH 2.5 phosphate buffer CNBr released collagen peptides move to cathode ahead of the endosmotic flow in a molecular mass reflecting sequence, the smaller ones in the front of the electropherogram, the larger at its end. As expected the migration time vs. molecular mass relationship is linear as shown in Fig. 4. However if the same sample is run in the 25 mM phosphate buffer at pH 4.5 containing 0.1% SDS, no peaks were observed within 1 h running time, while with reversed polarity the profile shown in Fig. 2B was observed. Similarly to Fig. 2 also here the sequence of eluted peptides reflects their molecular mass, the larger peptides being eluted first, the small ones at the end of the electropherogram. However the dependence of migration time vs. molecular mass bends up for peptides of smaller molecular mass as shown in Fig. 4. Comparison of migration times of individual CNBr-released peptides in the presence and absence of SDS in the background electrolyte is shown in Table 1. Further support for the idea that speeding up of the separation of collagen-derived peptides can be done by converting them into SDS-complexes has been

done by separating the CNBr-released peptides by slab gel electrophoresis, isolating some of them by the procedure described in Section 2 and running the isolates in pH 2.5 phosphate buffer (25 mM). The results are shown in Fig. 5. The span of the profile corresponds perfectly to what would have been predicted from the behavior of tissue collagenase released fragments from the blood vessel wall collagen exposed to hypoxic conditions: the peptides move in a molecular mass dependent manner, the small peptides preceding the larger ones. However, as shown in Fig. 6 the migration time vs. molecular mass dependence is curvilinear. As it emerges from the molecular mass vs. migration time plots of the collagenase released fragments and their comparison with migration of CNBr released fragments used as standards, the fragments released under hypoxic conditions *in vivo* have molecular masses of 34 000 and 64 000 respectively. This is in a good agreement with the image of tissue collagens being split in about three quarters of the parent molecule (calculated from its N-terminal by tissue collagenases [12]). In CE the presence of a third fragment of rel. mol. mass 17 000 was found. This may refer to a second cleavage site of this collagens in the collagen molecule as reported in [12]. The 34 000 rel. mol. mass fraction was isolated from the CE runs and partially sequenced: two sets of data were obtained revealing the presence of two amino acids in each sequencing step in a ratio of 1.82:1. This is quite understandable if one recalls the fact that the two constituting collagen  $\alpha$  chains are released simultaneously. Taking into account the  $\alpha 1$  to  $\alpha 2$  chain ratio of 2:1 the sequences IAGQ and LLGA were constructed; these corresponded perfectly the cleavage site of tissue collagenase of the two parent collagen  $\alpha$ -chains [17]. It was concluded that the 34 000 rel. mol. mass peak (no. 2) in the tissue collagenase fragments pattern is composed of two fragments, one stemming from the  $\alpha 1$ , the other from the  $\alpha 2$  chain. Consequently it can be predicted that peak no. 1 in this pattern corresponds to two fragments. Because the molecular size of fragments released both from the  $\alpha 1$  and  $\alpha 2$  chain are very close and since the separation both in the presence and absence of SDS in the background electrolyte is based on some mechanism reflecting the molecular size of the analytes, it is not surprising that the

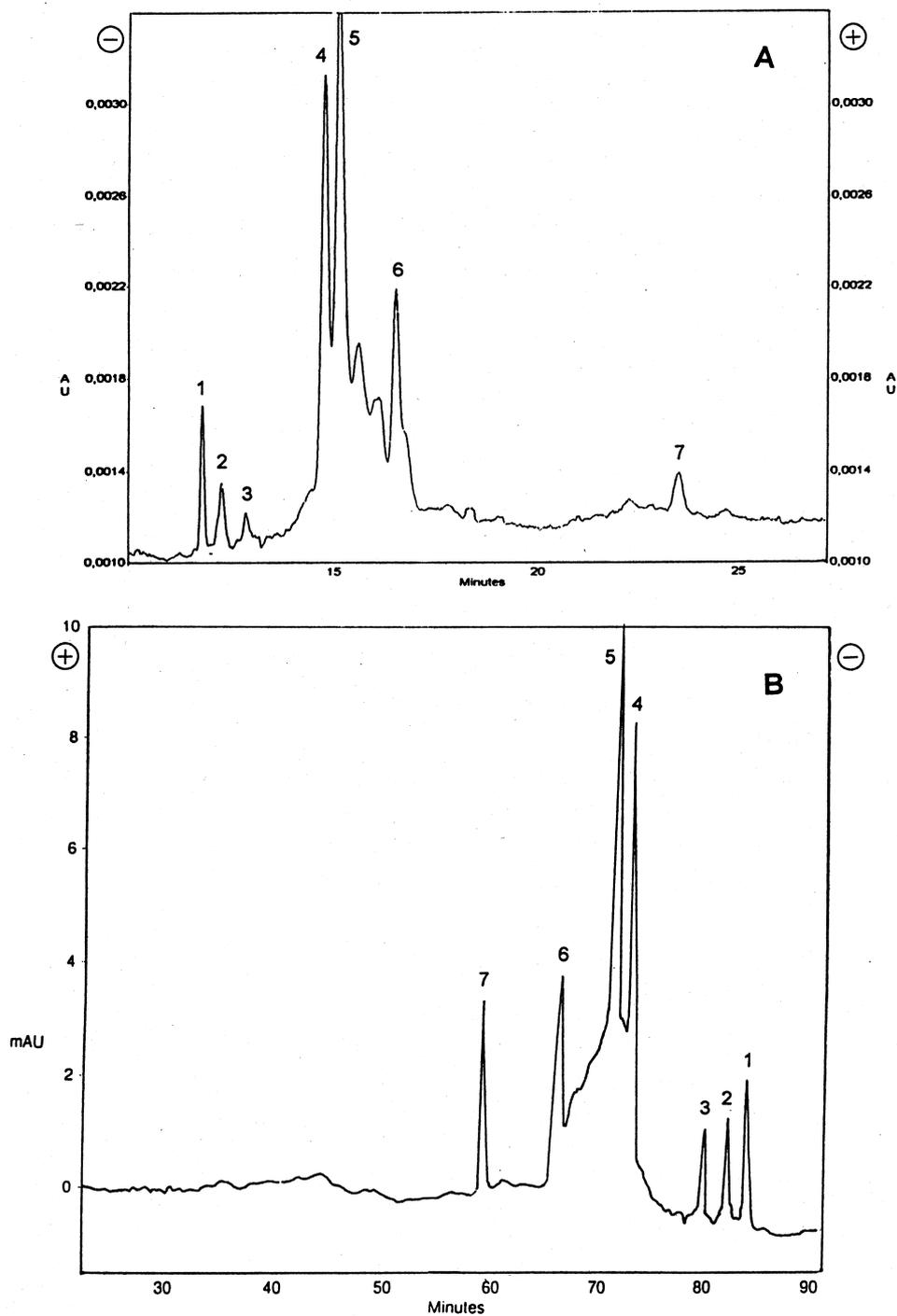


Fig. 3. Separation of the calibration set of collagen CNBr peptides. (A) CE in 25 mM phosphate buffer (pH 2.5), peptides prepared by CNBr cleavage; (B) CE in 25 mM phosphate buffer (pH 4.5), with 0.1% SDS. Peak identification: 1,  $\alpha_1(\text{I})\text{CB}_2$ , 2,  $\alpha_1(\text{I})\text{CB}_4$ , 3,  $\alpha_1(\text{III})\text{CB}_3$ , 4,  $\alpha_1(\text{I})\text{CB}_6$ , 5,  $\alpha_1(\text{I})\text{CB}_7 + \alpha_1(\text{I})\text{CB}_8$ , 6,  $\alpha_2(\text{I})\text{CB}_4$  and 7,  $\alpha_2(\text{I})\text{CB}_{3,5} + (\alpha_1(\text{III})\text{CB}_9)_3$ . Other conditions as specified in Section 2.

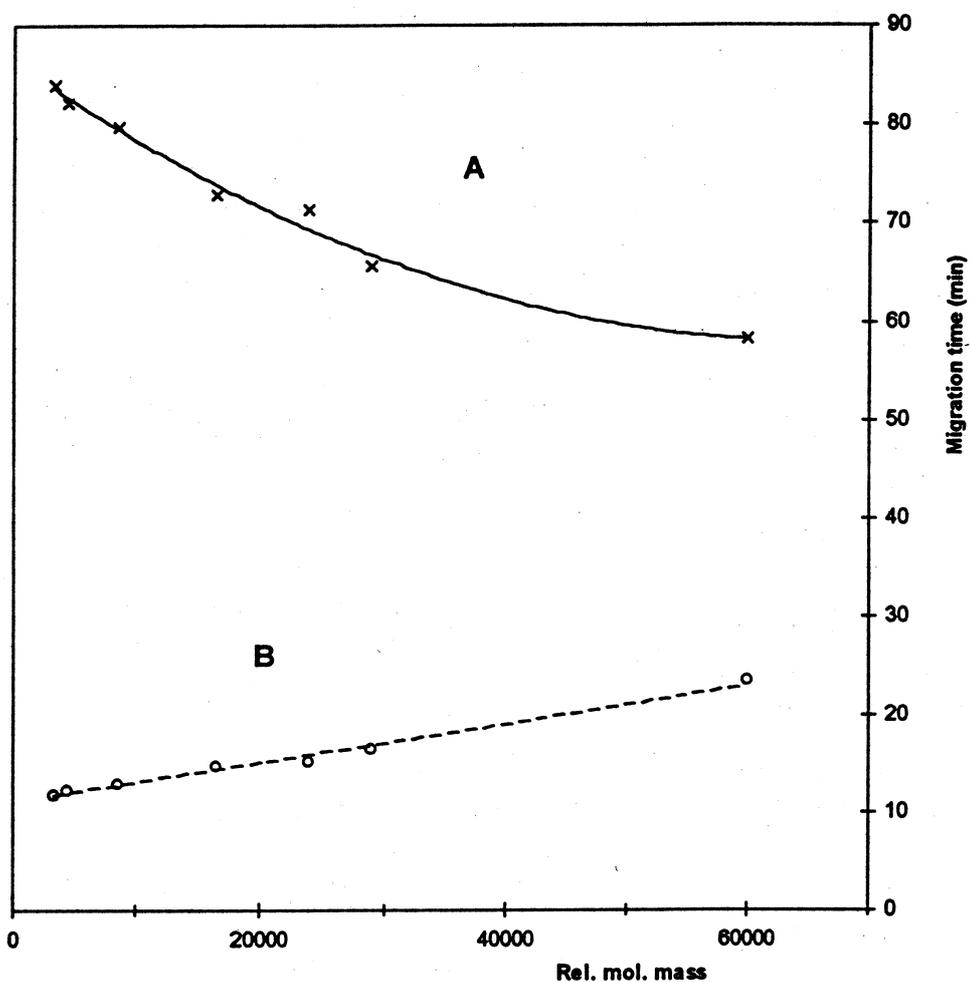


Fig. 4. Migration time vs. rel. mol. mass dependence as revealed by capillary electrophoresis in the presence (A) and in the absence (B) of SDS in the background electrolyte.

Table 1

Migration times and rel. mol. mass of CNBr released collagen peptides in capillary electrophoresis run at pH 2.5 (in the absence of SDS) and at pH 4.5 (in the presence of SDS)

No.	Peptide	Mol. mass	Migration time (min)	
			SDS complex	Bare peptides
1	$\alpha_1(\text{I})\text{CB}_2$	3300	83.91	11.78
2	$\alpha_1(\text{I})\text{CB}_4$	4600	82.17	12.28
3	$\alpha_1(\text{III})\text{CB}_3$	8600	79.78	12.89
4	$\alpha_1(\text{I})\text{CB}_6$	16 500	72.83	14.78
5	$\alpha_1(\text{I})\text{CB}_7 + \alpha_1(\text{I})\text{CB}_8$	24 000 + 24 000	71.30	15.11
6	$\alpha_2(\text{I})\text{CB}_4$	29 000	65.65	16.50
7	$\alpha_2(\text{I})\text{CB}_{3,5} + (\alpha_1(\text{III})\text{CB}_9)_3$	60 000	58.26	23.44

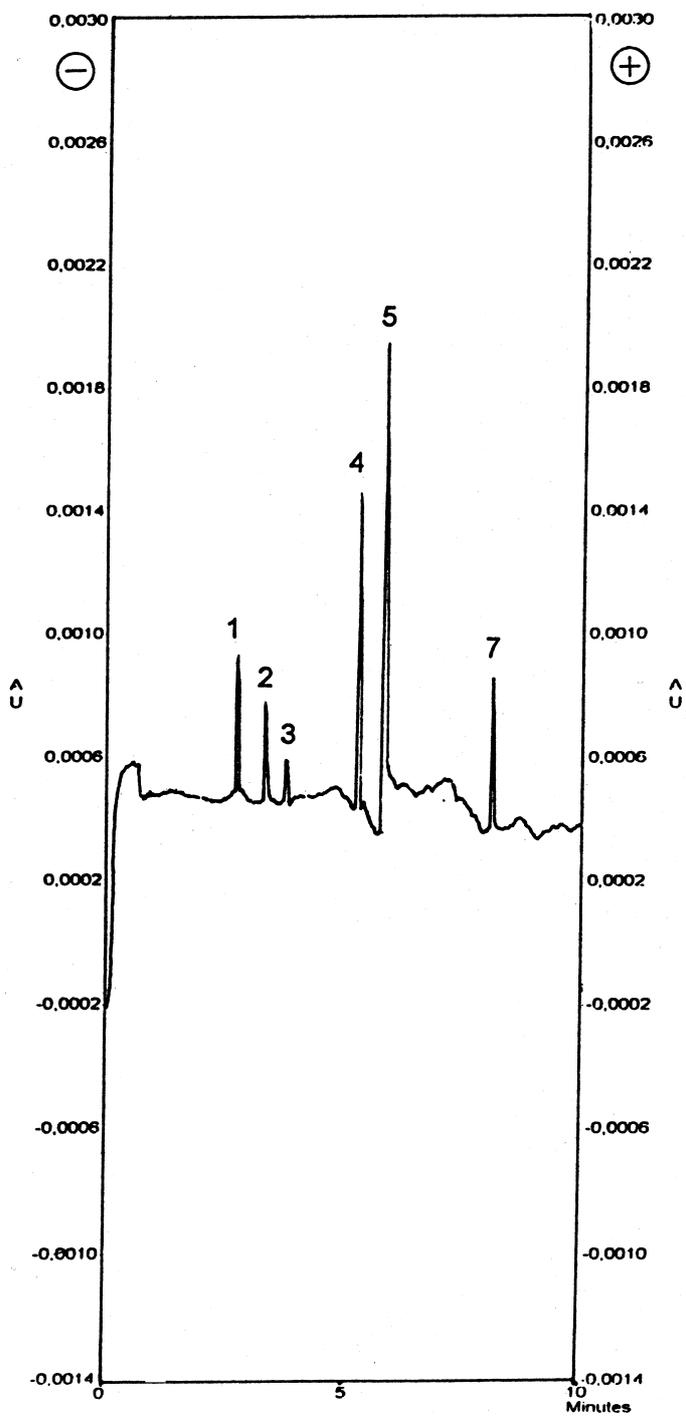


Fig. 5. Capillary electrophoretic separation of a set of collagen CNBr fragments isolated from SDS slab gel electrophoresis and run in the 25 mM phosphate buffer (pH 2.5). Peak identification as in Fig. 3; separation at 15 kV. Polarity of electrodes as indicated (normal polarity mode).

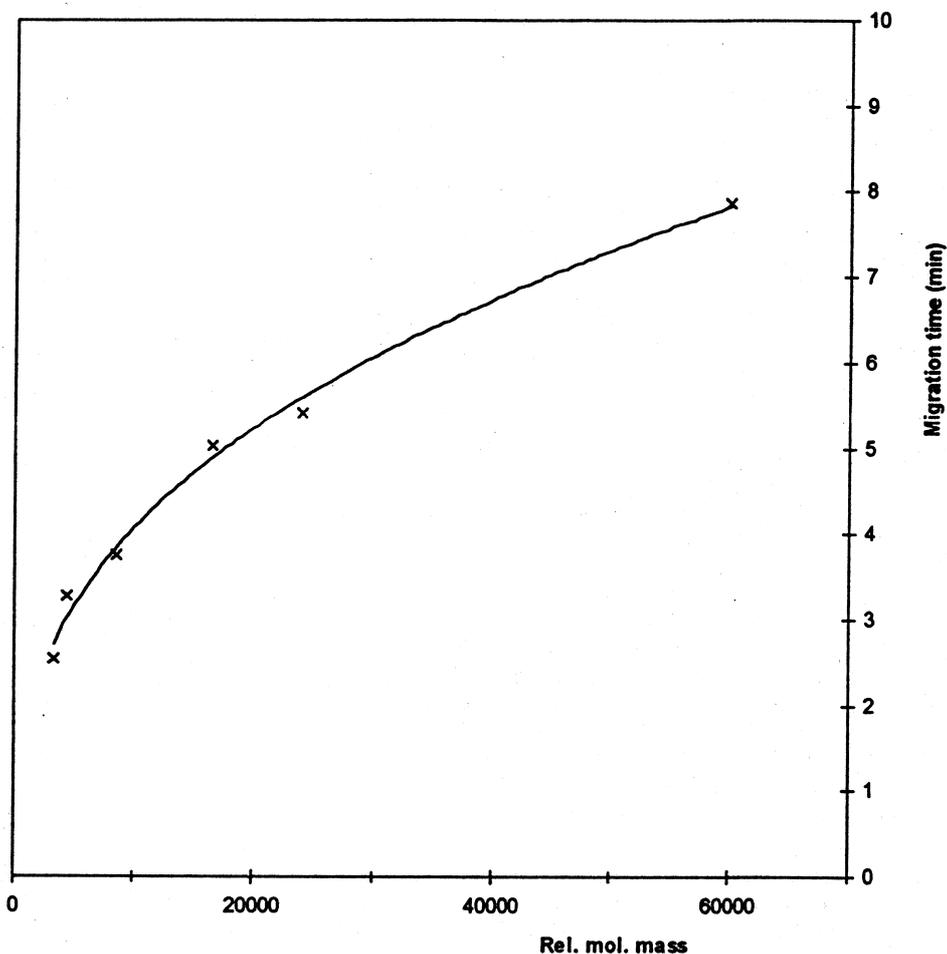


Fig. 6. Migration time vs. rel. mol. mass for CNBr peptides isolated from polyacrylamide gel SDS-electrophoresis and run at pH 2.5 in 25 mM phosphate buffer.

individual entities are not separated by either of the procedures investigated (pH 2.5 in the absence of SDS and pH 4.5 in the presence of the surfactant).

#### 4. Discussion

Micellar electrokinetic chromatography of proteins represents a new dimension in CE that makes use of hydrophobic and electrostatic interactions of protein analytes with the surfactant micelles present in the buffer medium for separation. As reviewed recently [3] a number of proteins have been successfully separated by this approach. Most of the separations

described use either SDS or cetyltrimethylammonium bromide as micelle forming agents. However, changing of the polarity of the electrophoretic system appears necessary according to the arrangement of the experiment.

In the present communication we have used SDS–collagen complexes both in the presence and absence of the micellar mobile phase for the separation of collagen derived peptides; the basic idea was to verify the molecular mass and identity of tissue collagenase released fragments in vascular connective tissue remodelling. Because of the well known differences in mobile phase estimation of collagens and their fragments when standard globular proteins

are used for calibration we used CNBr fragments of collagen of known molecular mass and sequence as calibration standards.

Polyacrylamide slab gel electrophoresis appears the method of choice for routine screening of collagen disorders in tissues because of its selectivity and because it allows working out of a large number of samples simultaneously. Though microsequencing of the separated zones in polyacrylamide gel is possible, the problem is how to assay the purity of the separated peptidic fragments and how to verify their molecular mass. Consequently it would be advantageous to use some additional separation step which, if possible, would additionally offer more reliable quantitation of the individual zones than stained polyacrylamide gel. There are theoretically two choices: either to use reversed-phase chromatography or CE. The application of the former technique is precluded to a large extent by its demands upon the sample amount, while the latter is rather straightforward. On the other hand, as demonstrated in our report, the results may be considerably influenced by whether or not a particular sample was subjected to the interaction with SDS at some of the preparative steps.

Two approaches were used in separating both the collagenase released fragments and the standard set of CNBr released peptides by CE, namely separation in phosphate buffer (pH 2.5) and separation in phosphate buffer (pH 4.5) made 0.1% with respect to SDS (supramicellar concentration of the surfactant). In both cases the peptides were separated in a molecular mass dependent manner, however, in the acid background electrolyte the small peptides migrated first, while in the more alkaline buffer the small peptides migrated last. The mechanism involved is apparently not a simple one and, perhaps, more than one type of interaction is involved. If one considers the number of free amino groups available per fragment, no correlation exists even when taking into account the size of the fragment. In a previous report we have postulated that the separation in acid media is based on the interaction of the peptides with the inner walls of the capillary, presumably by hydrophobic forces. This conclusion was derived from the analogy between the separation pattern of CNBr released peptides and the result of liquid column chromatography on reversed-phase [10]. It is

generally accepted that more hydrophobic entities are more retained upon reversed-phase columns. This means, per analogies, that the separation in acid media involves hydrophobic interactions with the capillary wall. However, if this is true, than complexation with SDS (which at the run buffer pH would be practically undissociated) would result in either lowering or abolishing the interaction between the solutes and the capillary wall. As shown in Figs. 2 and 5, this is indeed what happens. The migration being driven mainly by the negative charges of the individual components in the mixture is much faster, which means that the retaining force, whatever it is, must be smaller.

On the other hand, if the surfactant forms a micellar phase as is the case at pH 4.5, the members of the mixture are attracted to the micelles at a much higher affinity than to the capillary wall (the strong affinity of the separated peptides to SDS strongly favors this assumption) and, consequently, the peptides become attached to the micelles and negatively charged. This makes them move to the anode and the system must be run at inverse polarity to see the result. The fact that the molecular mass vs. migration time is curvilinear supports this image as it is unlikely that the larger peptides will bind proportionally more surfactant.

## 5. Conclusions

A system for the isolation of collagen fragments from blood vessels along with their identification and molecular mass estimation is described. The ultramicropreparation is based on dissecting the vessels, removing the non-collagenous proteins and solubilizing the collagenous core by pepsin. The released collagen parent  $\alpha$  chains and their fragments are separated for routine screening by SDS-PAGE and their identity and molecular mass estimates are verified in a subsequent CE run. This can be done either in the straight mode at pH 2.5 in a 25 mM phosphate buffer when the small peptides run ahead of the larger ones, however, the separation time is considerably shortened (roughly by a factor of three) compared to separations of collagen released peptides which have not come in contact with SDS during their preparation. For preparative purposes,

however, this approach is difficult to apply because the time differences between the appearance of individual peaks is quite short precluding effective accumulation of individual peaks in the cathodic vessel of the CE equipment. However, the separation can be also materialized at pH 4.5 in 2 mM phosphate buffer made 0.1% with respect to SDS. In this case the larger peptides run ahead of the smaller ones, however, the system must be run in the reversed polarity mode. In this case the differences between the migration time of individual components present are much larger and allow an easy accumulation of the individual compounds in the anodic vial. In concert with our previous communication [10] it is proposed that in pH 2.5 buffer the separation is based on the interaction of the solutes with the capillary wall, while in the more alkaline media in the presence of SDS micelles the leading partition mechanism is that of micellar electrokinetic chromatography.

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