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# Capillary electrophoresis of large cyanogen bromide peptides of fibre-forming collagens with special reference to cross-linking

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## Abstract

Cyanogen bromide (CNBr) peptides derived from collagen types I and III and having a relative molecular mass of more than 13 500 exhibit a linear relationship between molecular mass and migration time. This behavior is similar to the elution of CNBr peptides of collagen types I and III from wide pore (30 nm) C<sub>18</sub> reversed-phase columns. The electrophoretic procedure is very rugged and the linearity is preserved over the pH range 2.0–2.5, with a background electrolyte concentration of 20–100 mmol/l and an applied voltage of 8–25 kV [per 70 cm (effective length 63 cm)×75 μm I.D. capillary]. Modification of the inner capillary surface or addition of an organic modifier ruins the separation. The separation mechanism is apparently multimodal, as no correlation between the number of total amino acid residues forming a peptide, the number of proline and hydroxyproline residues or the number of glycines could be established. Also, there is no secondary structure involved, as the results with native and denatured peptides were the same. Application of this approach to reveal higher molecular mass peptides (~40 000) in rat tail tendons of two-year-old rats compared with three-month-old rats is presented.

**Keywords:** Peptides; Collagen

## 1. Introduction

There are basically two strategies that are used to reveal the biochemical changes underlying alterations in the collagen structure that accompany aging. One focuses on separating collagen chain polymers of the pepsin-solubilized insoluble collagen core, either by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or chromatographic procedures [1–3], the other follows the estimation of cross-linking amino acids (preferably lysinonor-leucine; both its glycosylated and non-glycosylated

forms) [3–7]. Although the progressive insolubility of fiber-forming collagens with age seems to be well documented, there is no evidence for an increased proportion of “physiologically” lysine-derived cross-links [8]. More recently, another enzymatically unregulated pathway that may lead to the insolubility of collagen with age and, under some pathological circumstances (e.g. diabetes), is derived from glycosylated lysine residues has been proposed (for review see [9–12]) and subjected to intensive investigation. It appears, however, that glycation may affect a number of lysine residues in the collagen molecule (particularly of collagen type I), however, *in vitro* studies reveal that only some of the glycosylated residues are sterically located in such a way that

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allows cross-linking to occur, perhaps accompanied by the formation of fluorescent compounds [13–15].

Localization of either type of cross-link within the fiber-forming collagen molecules (mainly types I, III and V) relies on the separation of collagen derived cyanogen bromide (CNBr) peptides, which is routinely done by PAGE [16]. However, quantitative evaluation of the arising peptide profile appears difficult because of different affinities of the individual peptides to the dyes used for staining (including silver staining procedures) [17], which is particularly pronounced in collagens subjected to glycation *in vitro*.

Although a procedure that allows the direct quantification of the three main fiber-forming collagens (type I, III and V), exploiting reversed-phase chromatography after tissue collagen cleavage (solubilization) by CNBr, has been published [18,19] and recently a fast procedure for revealing the proportion of collagen types in connective tissues (by exploiting capillary electrophoresis) has been reported [20,21], little attention has been paid to peptides other than marker peptides of individual fiber-forming collagens. The reason was that this methodology was aimed at revealing changes in the proportions of collagen types I and III in vascular stroma-related oxidative stress and age-related collagen polymerization was not considered.

Generally speaking, the marker peptides for individual collagen types belong to the family of low-molecular-mass species ( $M_r$  of less than 5 000) and their profile remains unchanged within a broad range of experimental conditions (voltage applied, background electrolyte (BGE) composition and concentration and temperature), although, of course, the migration times are shortened e.g. with buffer dilution, higher voltage applied or at higher temperatures. However, the same is basically true for the high-molecular-mass peptides ( $M_r$  values beyond 13 500), with the order in which they appear in the detection window being related to their relative molecular masses (the larger the peptide the longer the migration time). As a matter of fact, the profile of CNBr peptides of e.g. collagen type I polypeptide chains nearly copies the profile obtained with large pore size (300 Å) reversed-phase chromatography. Therefore, it was concluded that the predominating separation mechanism is based on hydrophobic

interactions between the peptides and the inner surface of the capillary wall. In capillary electrophoresis, the addition of an organic modifier to the background electrolyte or inner surface modification result in a distinct decrease in the resolution.

In contrast, there is a large free separation window available between  $\alpha_2\text{CB}_4$  ( $M_r$  29 000) and  $\alpha_2\text{CB}_{3,5}$  peptides ( $M_r$  60 000) (the last two peptides of the profile), where some of the cross-linking may occur. If, however, the cross-linked peptide has a molecular mass of around 25 000, it will not be revealed as it is reasonable to assume that it will probably be hidden in the dominating double peak that emerges close to the middle of the profile.

It was the aim of this investigation to reveal whether peptides of molecular masses between 29 000 and 60 000 can be obtained from old connective tissue (rat tail tendons) and to confirm this finding by comparison with the results obtained by reversed-phase chromatography and SDS gel electrophoresis.

## 2. Experimental

### 2.1. Capillary electrophoresis

All separations were performed on a SpectraPhoresis 500, TSP-instrument, obtained from Watrex (Prague, Czech Republic) using PC 1000 software, version 2.6. The capillary used was uncoated and had the dimensions 70 cm (63 cm to the detector)  $\times$  75  $\mu\text{m}$  I.D. Separations were run with 20–100 mM phosphate buffer that was purchased either from Bio-Rad (Richmond, CA, USA, catalogue No. 148-5010 with polymeric modifier) or was prepared in the laboratory; the separation voltage used was 15–25 kV. If not specified otherwise, separations were run at 15 kV [high voltage (anode) applied to the injector end of the capillary], yielding 30–70  $\mu\text{A}$  of current, depending on the temperature (runs were performed both at 25 and 50°C, to determine the influence of the denaturation of large peptides present in the mixture) and on BGE concentration. Depending on the protein content in the sample, hydrodynamic sample application for 1–8 s (vacuum 10.3 kPa) was used. Detection was by UV absorbance at 200 nm. Benzylalcohol (Sigma, St.

Louis, MO, USA) was used as the endosmotic flow marker and changes of within  $\pm 2\%$  of the migration time of individual peptide peaks was considered sufficient for both within-day and day-to-day variability. A peptide concentration of 400  $\mu\text{g}/\text{ml}$  of the protein was used routinely.

## 2.2. Preparation of CNBr peptides

CNBr peptides were prepared from collagen type I (Sigma) or from collagen types III and V that were prepared in the laboratory from rat tail tendons following limited pepsin digestion and selective salt precipitation according to established procedures [22]. These samples were treated with CNBr without collagen chain separation in 70% formic acid as described by Scott and Veis [16]. This procedure yields a considerable proportion of uncleaved peptides which emerged close to the joint peak of  $\alpha_1(\text{I})\text{CB}_7$  and  $\alpha_1(\text{I})\text{CB}_8$ . Lyophilized CNBr peptide preparations were redissolved in Milli-Q water (Millipore, Bedford, MA, USA), centrifuged and lyophilized again.

## 2.3. Isolation of individual peptides

Isolation of marker peptides was based on a set of chromatographic procedures involving ion-exchange chromatography, reversed-phase chromatography and gel permeation separations on Ultrogel and Bio-Sil. The general strategy employed ion-exchange separation as the first preparative step, followed by either Ultrogel AcA chromatography or reversed-phase separation. Purity of the fractions obtained was checked by PAGE and, where sufficient material was available, also by amino acid analysis.

Carboxymethyl (CM)-cellulose chromatography, generally following the procedure of Butler et al. [23], was used. A column (15 $\times$ 1.5 cm) was packed with CM-cellulose (CM 52; Whatman, Clifton, NJ, USA) and operated at 42°C. The column was equilibrated with 0.02 *M* citrate buffer, pH 3.6, and eluted at 100 ml/h with a linear ionic strength gradient from 0.02–0.14 *M* (2000 ml in total). The amount of peptides loaded was about 100 mg, the effluent was monitored at 220 nm and corresponding fractions from five runs were pooled and lyophilized.

Gel permeation chromatography followed as the next step: Two 300 $\times$ 7.5 mm columns in tandem were used (Bio-Sil, SEC-125, Bio-Rad); elution was isocratic with 0.5 *M* guanidine HCl–50 mM Tris, pH 7.5, at a flow-rate of 0.6 ml/min. Effluent was monitored at 220 nm (method is described by Miller et al. [19]). With larger peptides (above 25 000), the gel permeation step was performed with an Ultrogel AcA-54 (LKB, Bromma, Sweden) column (400 $\times$ 16 mm), at 20°C and elution at 40 ml/h with 0.02 *M* citrate buffer, pH 3.6. The effluent was monitored at 230 nm and was recycled three times.

## 2.4. Reversed-phase chromatography

A Vydac TP 201 column (250 $\times$ 4.6 mm, 30 nm pore size) (Separations Group, Hesperia, CA, USA) was used with a linear gradient from 12.8–44.8% acetonitrile in water at a flow-rate of 1 ml/min over 1 h. Heptafluorobutyric acid (0.01 *M*) was used as the ion-pairing agent. The equipment used was from Waters and comprised two model 6000 A pumps, a 660 model gradient programmer and a U6K injector. Detection was done by means of a UV-Vis spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) set to 200 nm. The procedure was essentially the same as that of Van der Rest and Fietzek [24]. This procedure was used to compare the molecular masses of separated CNBr peptides; in some cases, it was used to verify the nature of a particular peptide separated in the two previously described chromatographic steps. Enriched fractions of some CNBr liberated peptides, namely  $\alpha_1(\text{I})\text{CB}_6$ ,  $\alpha_1(\text{I})\text{CB}_7$  and  $\alpha_1(\text{I})\text{CB}_8$  which can be obtained directly by reversed-phase chromatography.

## 2.5. Gel electrophoresis

PAGE was used to check the purity of a particular peptide and was done according to the procedure described by Novotná et al. [20]. In some cases, even the three-step procedure did not yield a pure peptide. In such cases, the particular peptide was prepared by extracting the zone from the polyacrylamide gel and the behavior of such an isolate was confronted with the peptide prepared by chromatographic procedures.

## 2.6. Chemicals

All chemicals used were either of analytical grade or the highest purity available. Formic acid (88%) was obtained from Lachema (Brno, Czech Republic), 2-mercaptoethanol was from Sigma. Sodium formate was purchased from Baker (Phillipsburg, NJ, USA). Phosphate buffer, pH 2.5, was either purchased from Bio-Rad or prepared from sodium monophosphate (Lachema) with the pH adjusted using phosphoric acid (Lachema). Ammonium bicarbonate, CNBr and guanidine hydrochloride (electrophoresis grade) were from Sigma. All solutions were prepared in Milli-Q water.

## 3. Results and discussion

During previous experiments, we observed that in acidic buffers (pH 2.5) CNBr peptides derived from collagen are separated in order of increasing molecular mass. In this report, we summarize our results obtained with both isolated peptides and peptides in CNBr collagen hydrolysate without further separation of the peptides that arose. As shown in Fig. 1, the linear relationship between molecular mass and migration time is preserved over a wide range of background buffer concentrations (20–100 mmol/l). The molecular mass vs. retention time appears to be steeper with more dilute buffers, which corresponds

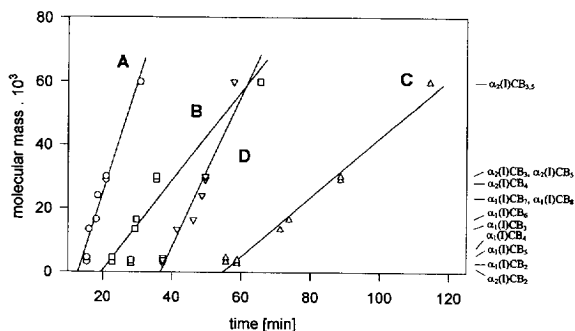


Fig. 1. Correlation between the relative molecular masses of collagen type I CNBr peptides and their migration times. Voltage applied, 15 kV; temperature, 50°C; capillary, untreated, 70 cm (effective length 63 cm) × 75 μm I.D. Buffer, phosphate buffer, pH 2.5; A=20 mM buffer; B=50 mM buffer; C=100 mM buffer; D=reversed-phase HPLC. For nomenclature of CNBr peptides see Ref. [9].

to faster migration (larger electroosmotic flow) at lower buffer concentrations.

As expected variations in the voltage applied [8–25 kV per 70 cm (effective length 63 cm) capillary] did not influence the linearity of the relationship between relative molecular mass and migration time.

There are several factors that may cause this unexpected behavior; the first is the unusual amino acid composition (with few hydrophobic and charged amino acids in the molecule and the helical conformation of polyproline II) that even CNBr peptides tend to occupy in solution [25,26]. In order to answer the question of the extent to which the helical conformation may be responsible for this behavior, we have boiled the samples for 1 min before analysis and have performed the electrophoresis at 50°C. This treatment had no effect upon the retention time of individual peptides and the relationships in all types of buffers investigated (20, 50 and 100 mmol/l) remained the same.

Next, we selected a number of chemical parameters and investigated the extent to which they correlate with the migration time observed. The data obtained are summarized in Table 1. In particular, the total number of amino acids, the number of Pro and Hypo residues and the number of glycine residues were taken into account. The total number of amino acids was considered because it has been shown that, in gel electrophoresis, the migration of collagen α-chains, as well as their fragments, correlates better with the total number of amino acids rather than with the molecular mass of the separated species. The correlation coefficients were high, with all the parameters investigated. The lowest correlation found was between migration time and the content of Pro+Hypo. Similar conclusions can be drawn if correlations to the molecular mass of the peptide are related to the electrophoretic migration velocity of individual peptides, taking into account the small changes in the migration times of the individual peptides and the electroosmotic flow marker.

In the next step, we compared the electromigration behavior of individual collagen CNBr peptides with their behavior during reversed-phase chromatography. In this comparison, we followed the method of Van der Rest et al. [18,24], using a 30-nm pore size reversed-phase C<sub>18</sub> packing. As proposed by Van

Table 1

Correlation between various parameters [ $M_r$ , total number of amino acids, number of proline and hydroxyproline (Pro+Hypro) and glycine (Gly) residues] and migration time (retention time) in capillary electrophoresis and reversed-phase chromatography of collagen type I CNBr peptides

Parameter	<i>a</i>	<i>b</i>	<i>r</i>	<i>r</i> <sup>2</sup>
<i>Capillary electrophoresis</i>				
<i>100 mM buffer</i>				
$M_r$	-50858	930	0.991	0.981
Amino acids	-571	10.5	0.990	0.981
Pro+Hypro	-100.2	1.9	0.983	0.966
Gly	-193.8	3.5	0.989	0.978
<i>50 mM buffer</i>				
$M_r$	-27216	1400	0.942	0.887
Amino acids	-302.8	15.7	0.937	0.878
Pro+Hypro	-50.6	2.8	0.919	0.844
Gly	-104.5	5.3	0.942	0.887
<i>20 mM buffer</i>				
$M_r$	-44672	3491	0.975	0.951
Amino acids	-492.9	38.9	0.972	0.946
Pro+Hypro	-78.6	15.3	0.952	0.907
Gly	-169.1	13.2	0.972	0.945
<i>Reversed-phase chromatography</i>				
$M_r$	-86694	2366	0.966	0.934
Amino acids	-984.0	26.8	0.974	0.948
Pro+Hypro	-180.8	5.0	0.984	0.969
Gly	-331.9	9.0	0.969	0.940

Linear relationships of the  $y=a+bt$  type;  $y$  is the investigated parameter,  $t$  is the migration (retention) time,  $a$  and  $b$  are experimentally determined constants.

der Rest et al. [18,24], we used heptafluorobutyric acid as the counter-ion, because it would increase the hydrophobicity of the peptide through ion-pairing [27]. The larger pores had a favorable effect on resolution and the more hydrophobic counter-ion increased both the resolution and the retention time. The authors stated that although the set of peptides eluted according to increased molecular mass, no clear-cut relation between molecular mass and retention time was observed. In our hands, however, a linear relationship was observed (as shown at the bottom of Table 1). Although the correlation between retention time and relative molecular mass was lower than in the electrophoretic separations, it was still quite high. On the other hand, it is worth noting that, in the chromatographic separation, the highest correlation was between the number of Pro+Hypro residues and the retention time. However, the

significance of this finding appears questionable in view of other results obtained by capillary electrophoresis. Smolenski et al. [28] also noted a relationship between molecular mass (and the hydrophobicity of peptides) and migration time, when separating collagen CNBr peptides by reversed-phase HPLC.

The difference between our finding and that of Van der Rest et al. [18,24] can be explained rather simply: Van der Rest et al. [18,24] took all of the liberated peptides into consideration, while we have experimentally verified that both in capillary electrophoresis and wide pore reversed-phase silica gel chromatography the linear relationship applies to peptides with molecular masses that are higher than 13 500; the low-molecular-mass peptides exhibit an unpredictable behavior that neither follows Offord's relationship (seen in alkaline media) nor the molecular mass dependence (seen in acidic media) which, as stressed before, is applicable to larger peptides only. From our data it follows that (1) a certain minimum peptide length is needed to observe the molecular mass (total amino acids)-dependent migration and (2) this molecular mass-dependent migration (retention in HPLC) is the inherent property of the whole peptide, reflecting the unique amino acid composition of type I collagen. The secondary structure (helix formation) does not have an effect on electromigration (or chromatographic) behavior.

The slight differences in correlating individual parameters in capillary electrophoresis and reversed-phase chromatography can be attributed to the differences in the experimental arrangement of both methods, namely the presence of pores (representing a steric hindrance though large enough) in RP-HPLC and the addition of a hydrophobic counter-ion (heptafluorobutyric acid) in the chromatographic separation. It is noticeable that in reversed-phase chromatography, the addition of an organic modifier to the BGE improves the shape of the peaks and destroys the linearity of the molecular mass–migration time dependence. The large peptides, like  $\alpha_2(I)CB_{3,5}$ , move much faster than would be expected. This indicated that hydrophobic interactions or conformational changes due to the presence of a less hydrophilic BGE must be involved in the apparently multifactorial interaction of the CNBr peptides with the capillary wall. Indeed, if a surface-modified

capillary (polyacrylamide coated) is used, the selectivity is lost, although the larger CNBr peptides still move more slowly than the smaller ones (data not shown). The main advantage of the capillary electrophoretic separation of collagen CNBr peptides in acidic media (using acidic buffers and an untreated capillary) lies in the large separation window between the  $\alpha_2(\text{I})\text{CB}_4$  peptide ( $M_r$  29 000) and the joint peak of  $\alpha_2(\text{I})\text{CB}_{3,5} + (\alpha_1(\text{III})\text{CB}_9)_3$  ( $M_r$  values of 60 000 and 62 300, respectively), which is not available in the reversed-phase chromatographic separation. This offers the opportunity to determine the presence of cross-linked CNBr peptides (as shown in Fig. 2). Fig. 2A shows the profile of a CNBr peptide mixture obtained from rat tail tendons

of two-year-old rats, while Fig. 2B represents an analogous separation obtained from using six-month-old rats. There are two peaks seen at 47.6 and 49.2 min, respectively, in the rat tail preparations from the older rats, each of which comprise about 1% of the total peak area. In addition, we have observed the presence of a small peak (not quantifiable) at an elution time of around 38 min, which was absent in the preparations obtained from the younger counterparts.

The quantifiable peptides should have molecular masses of 39 900 and 43 600, respectively. If these were composed of two cross-linked peptides, they could be attributed e.g. to an  $\alpha_1(\text{I})\text{CB}_6$  dimer ( $M_r$  40 400) and an  $\alpha_1(\text{III})\text{CB}_5$  dimer ( $M_r$  43 800).

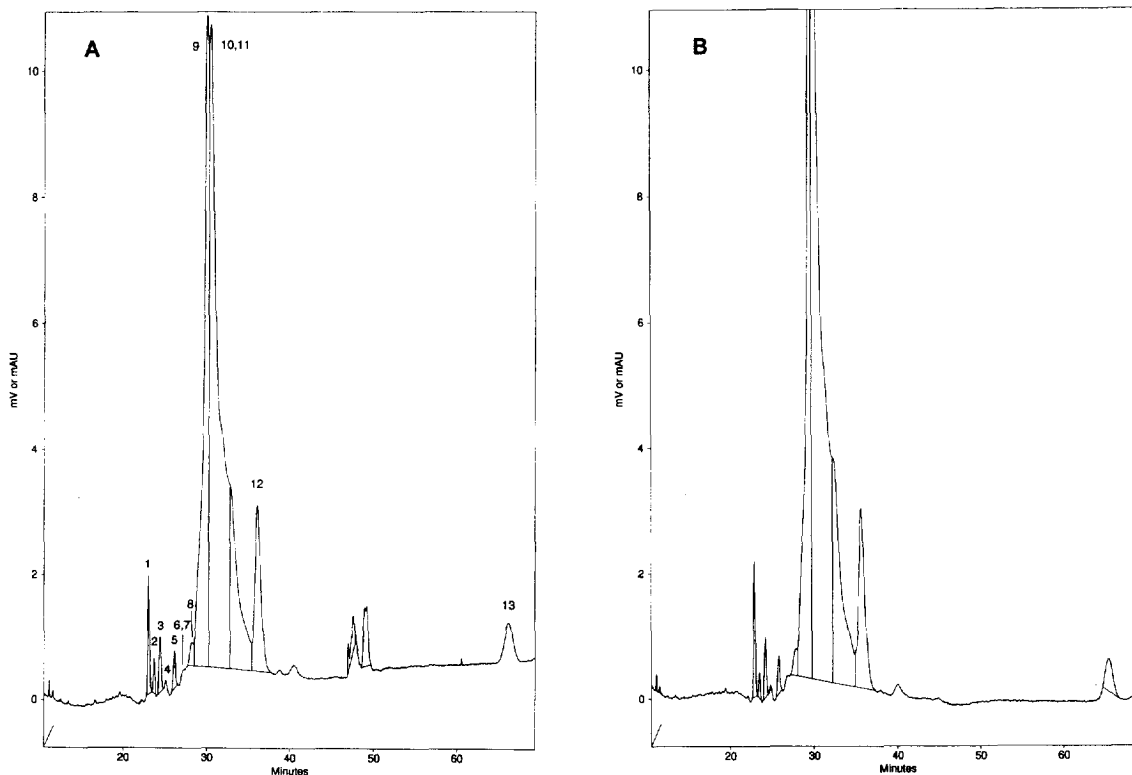


Fig. 2. Comparison of CNBr peptides obtained from rat tail tendons indicating the presence of a peptidic material with approx.  $M_r$  values of 39 900 and 43 600, respectively, and each comprising about 1% of the peak area in the preparations obtained from old (eighteen-month-old) rats. Separation conditions: 15 kV, 50°C, 50 mM buffer; (A) old rats (eighteen months), (B) young rats (six months). Note the presence of a small amount of peptide just before a migration time of 40 min in the sample prepared from the tail tendons of old rats ( $M_r \approx 32\,000$ ). Peak identification: 1 =  $\alpha_1(\text{I})\text{CB}_2$ ; 2 =  $\alpha_1(\text{I})\text{CB}_4$ ; 3 =  $\alpha_2(\text{I})\text{CB}_1$ ; 4 =  $\alpha_1(\text{V})\text{CB}_1$ ; 5 =  $\alpha_1(\text{III})\text{CB}_2$ ; 6 =  $\alpha_1(\text{I})\text{CB}_3$ ; 7 =  $\alpha_2(\text{I})\text{CB}_2$ ; 8 =  $\alpha_1(\text{I})\text{CB}_3$ ; 9 =  $\alpha_1(\text{I})\text{CB}_6$ ; 10 =  $\alpha_1(\text{I})\text{CB}_7$ ; 11 =  $\alpha_1(\text{I})\text{CB}_8$ ; 12 =  $\alpha_2(\text{I})\text{CB}_4$  and 13 =  $\alpha_2(\text{I})\text{CB}_{3,5} + (\alpha_1(\text{III})\text{CB}_9)_3$ . The identity of peaks in (B) is evident by comparison with peaks in (A). For nomenclature of CNBr peptides see Ref. [9].

Further investigation and, perhaps, combination with other techniques is needed to confirm this interpretation.

The success of the electrophoretic separation of CNBr collagen fragments is based on the appropriate amount of the sample being loaded into the capillary. The first indication of excessive sticking of collagen peptides to the capillary is current instability. This, however, can be avoided by appropriate washing of the column in-between runs (5 min with run buffer, 2 min with 0.1 M H<sub>3</sub>PO<sub>4</sub>, 2 min with run buffer, all at 50°C). It is advisable to let the electrophoresis run for about 1 h after every three runs. We have also observed that clogging and irreproducible sticking of peptide material occurs more frequently with capillaries that have been used for some time.

#### 4. Conclusions

It has been demonstrated that there is a linear relationship between relative molecular mass and migration time for collagen type I and collagen type III CNBr peptides. This linear relationship is valid within the pH range 2.0–2.5 (data not shown) and with an applied voltage of 8–25 kV on a 70-cm (effective length 63 cm) capillary and with a BGE concentration ranging from 20 to 100 mmol/l. Regarding the separation mechanism, the interaction with the capillary wall is probably multimodal, involving more than one type of interaction. For instance, hydrophobic interactions are partly involved because addition of organic solvent to the BGE (see [21]) changes the elution order of peptides (speeds up that of the very large peptides); the mechanism must involve the capillary surface, as its modification (with e.g. polyacrylamide) ruins the separation. On the other hand, we were unable to show a correlation between this behavior and the number of Pro+Hypro residues or the glycine content of the separated peptides. The behavior of collagen CNBr peptides in capillary electrophoresis and reversed-phase chromatography is very similar, however, the linearity between molecular mass and migration (retention) times applies to peptides of  $M_r$  13 500 and larger. The advantage of the electrophoretic approach is in the large empty separation window between  $M_r$  29 000 and 60 000, an area

where the occurrence of cross-linked peptides can be expected. This is demonstrated on the collagen CNBr peptide profiles obtained from the tendons of old and young rats.

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