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Review

Capillary electromigration methods for the study of collagen☆

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Abstract

This review paper gives an overview of capillary electromigration methods used in the analysis of collagen. Analyses of the parent chains as well as of the bromcyane and collagenase fragments of collagens are presented. Methods include capillary zone electrophoresis, capillary gel electrophoresis, micellar electrokinetic chromatography as well as combinations of HPLC and capillary electrophoresis, and capillary electrophoresis with mass spectrometry.

Keywords: Collagen; Capillary electrophoresis; Review

Contents

1. Introduction

The term collagen applies to a broad group of proteins. They are a family of extracellular matrix proteins that play a dominant role in maintaining the structure of various tissues and also have many other important functions (for example, adhesion, tissue remodelling) [1,2].

Collagens possess some typical features—they consist of three polypeptide chains (called α-chains) and contain at least one domain composed of a repeating tripeptide sequence: -Gly-X-Y-. These three chains are identical in some collagens, in others the molecules contain two or even three different α chains. The protein chains are coiled together into a left-handed helix and are then wound around a common axis to form a triple helix

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with a shallow right-handed superhelical pitch, and so the overall structure is a rope-like rod. The typical presence of glycine at every third position is essential for this packing to a coiled-coil structure and is one of the ways to determine the presence of collagen in a tissue sample. Any amino acid other than glycine can be present in the X and Y positions, but proline is often found in the X position and 4-hydroxyproline in the Y position. The 4-hydroxyproline plays a particularly important role, because these residues are essential for the stability of the triple helix. All collagens also have non-collagenous domains.

It should be mentioned that collagens are the most abundant proteins in the human body, constituting approx. 30% of its protein mass. At present, there are at least 27 known collagen types in vertebrates, containing a total of 42 distinct α chains, and more than 20 additional proteins have collagen-like domains, as shown in Fig. 1 [2]. Besides α-chains there are also β chains (dimers of α-chains) and γ chains (trimers of α-chain). It should be also stressed that collagen fibrils often contains more than one collagen type (α-chain). There are known cases when type I collagen fibrils contain also types III, V and XII, or fibrils of collagen type II can contain types IX and XI. It means that in the same tissue can be find not only fibrils of different collagen types but also "mixed" fibrils formed from various types. As it was mentioned, collagen family is a relatively broad group of proteins with a various molecular weight, when the molecular weight of α-chain of the most common type I is around 100,000 and its fibril (triple helical structure) is 300,000.

Individual collagen α-chains can be polymerised either by physiological cross-links (lysinonorleucine, hydroxylysinonorleucine, an aldol condensation product, pyridinoline) or by non-physiological cross-linking agents (typically aldehydes). Pathological crosslinking can be typically caused by interaction with

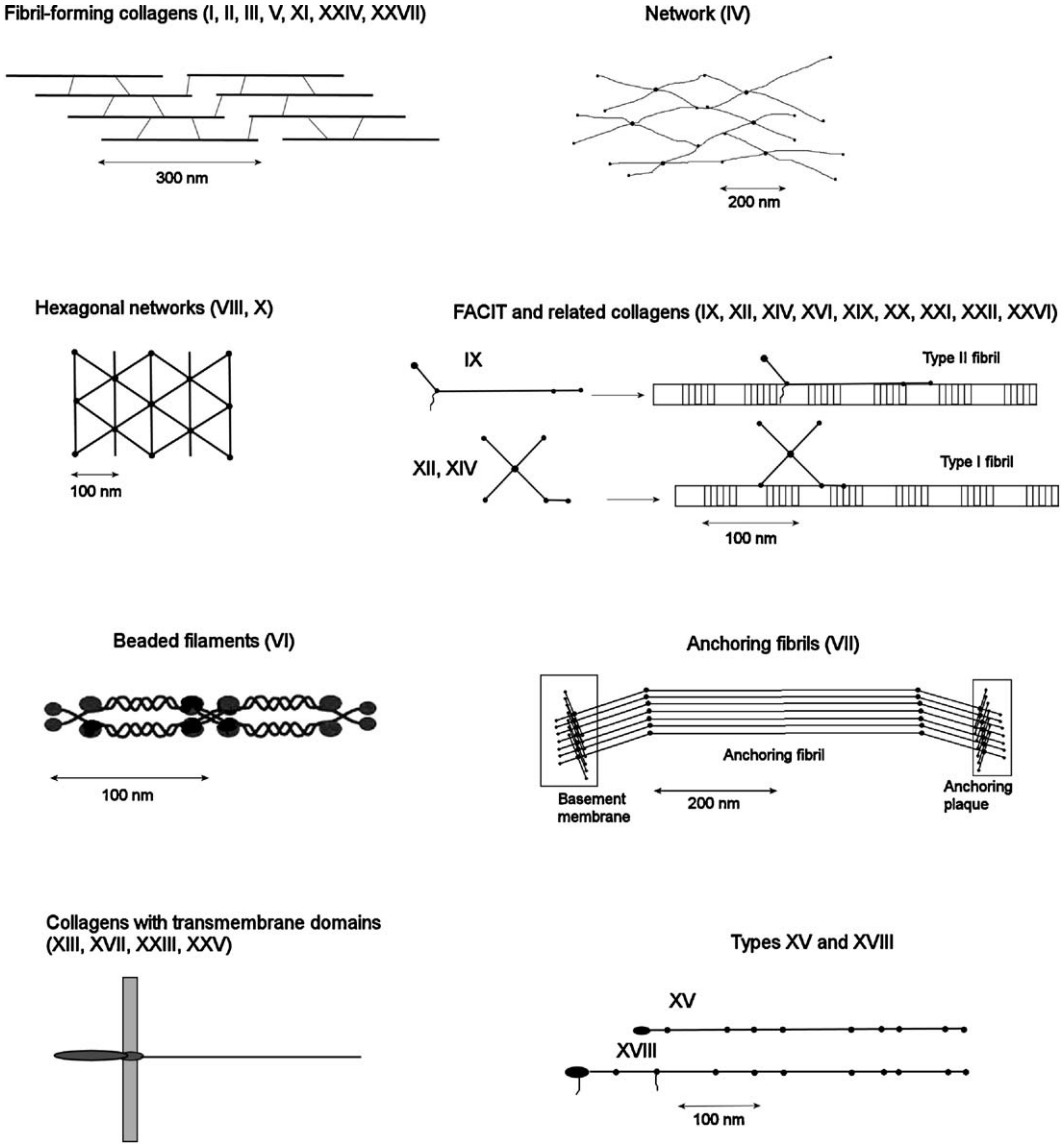

Fig. 1. Schematic display of various collagens' supramolecular assemblies. There are 27 types of collagen and more than 20 additional proteins have collagen-like domains (not shown). Abbreviation: FACIT, fibril-associated collagens with interrupted triple helices. Based on scheme previously published by Myllyharju and Kivirikko [2], with permission from Elsevier.

reducing sugars or the oxidation products of unsaturated lipids (monotopical interactions are also possible).

The most common types of collagens occur in fibres and networks. These proteins are poorly soluble (if at all), are found in many tissues such as connective tissue and have a slow metabolic turnover. This is the reason why they are more susceptible to some enzymatic or nonenzymatic posttranslational modifications.

Polymerised fibril-forming collagens (whether polymerised physiologically or non-physiologically) are insoluble and their solubilization is routinely performed either by mild pepsinization, in which short terminal regions possessing the polymerisation sites (cross-links) are cleaved off, or by CNBr cleavage, which results in a limited fragmentation of the parent α-chains as mentioned already. Tissue collagenases (which unfortunately are difficult to obtain) split the triple-helical structure two-thirds of the way from its N-terminus; bacterial collagenases (from *Clostridium histolyticum)* are far less specific, they cleave the sequence in small fragments (mostly tripeptides) and are therefore of little use in structural studies.

The investigation of these proteins could focus on their intact α-polypeptide chains or on their fragments (after cleavage). The most traditional methods for the analysis of collagens are slab gel electrophoresis, low-pressure and high-performance liquid chromatography, but the performance of these methods is low. Capillary electromigration methods are more efficacious but the properties of collagens, such as their hydrophobicity or the similarity of structure of individual peptides, make the separation difficult. It is probably for this reason that these methods are not frequently used for the study of collagens [3–6]. The review paper presented here will give an overview of the methods exploiting capillary electromigration methods that are suitable for the separation of collagens such as capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC), capillary electrochromatography, and a combination of capillary electrophoresis with HPLC and with MS.

This review paper is dedicated to Professor Zdenek Deyl, who developed many advanced analytical methods for the study of collagens and their modifications in connective tissues. Unfortunately he died in February 2005. He was not merely an outstanding scientist but also a notable teacher and good friend. We all miss him not only as an eminent figure in analytical science but also for his kind-hearted personality.

2. Parent chains

2.1. Capillary zone electrophoresis

In principle, in the analysis of collagen chains by capillary zone electrophoresis, very dilute buffers (typically 2.5 mM sodium borate, pH 9.2) or acidic buffers (pH ∼ 2.5, about 25 mM buffer) can be used.

In the one of the first publications about the capillary electrophoresis of collagen (if not the first), Deyl et al. [7] demonstrated that collagen types I, II, V, IX and XI consisting of α-chains and their chain polymers can be separated in a 2.5 mM

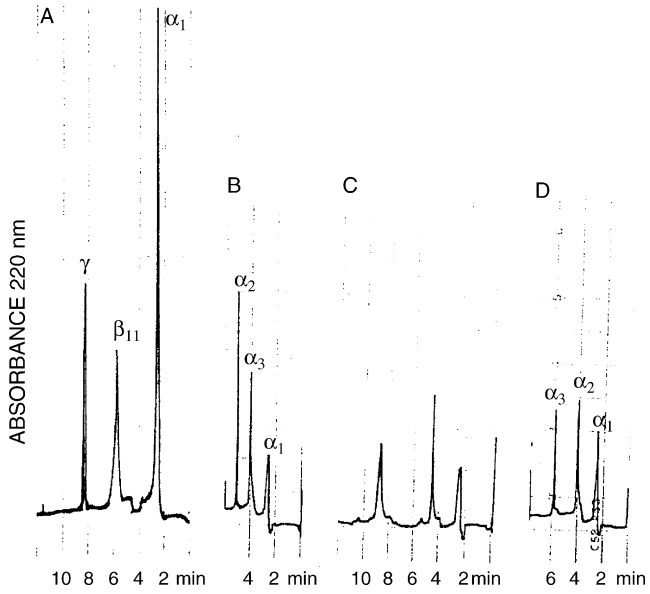

Fig. 2. Capillary zone electrophoretic separation (2.5 mM sodium tetraborate buffer, pH 9.2) of collagens (A) type II, (B) type V, (C) type IX and (D) type XI. Reprinted from Deyl et al. [7], with permission from Elsevier.

sodium tetraborate buffer at pH 9.2 in less than 15 min in a 50 cm × 100 μm fused silica capillary (Fig. 2). The separation was extremely sensitive to capillary overloading. When overloading occurred, poorly reproducible electropherograms were obtained. The reason for this irreproducibility is probably adherence to the capillary wall, as was determined at a later stage [8]. When the amount of sample used is kept within specific limits and the separation is run at sufficiently high pH (9.6) these effects can be prevented. The separation of groups of α, β and γ can also be achieved in a phosphate buffer (at the same pH) but resolution was lost—it was impossible to separate α_1 from α_2 chains and β_{11} from β_{12} chains. The similar effect was observed if the background electrolyte contained 0.5 mM sodium dodecyl sulphate (SDS) when the peaks of α, β and γ fractions only were possible to see. It was stated that the separation efficiency of the collagen chains is influenced by the presence or absence of a submicellar concentration of SDS in the background electrolyte. As was determined later, at higher (supramicellar) concentrations of the negatively-charged surfactant, all fractions migrate as a single broad zone.

Riis [9] used capillary zone electrophoresis at acid buffer (100 mM phosphate buffer, pH 2.5; with capillary 50 cm long, probably fused silica, and i.d. 50 μm) for analysis of soluble collagen from mink skin. There were analysed two extracts of skin—acid (0.5 M acetic acid) and NaCl (1 M). Author demonstrated that the amount of soluble collagen declines with age. Only one type of collagen (type I) was determined by capillary electrophoresis and separation shows only one peak of this type (without separation to tri-, di- and monomers).

2.2. Capillary gel electrophoresis

Capillary gel electrophoresis is nowadays routinely used and is a commercially available method for determining the

molecular mass of proteins/polypeptides. For this reason, it is not surprising that this method can be used for the separation of collagen chains and their polymers.

The separation of collagen type I α-chains and chain polymers β (dimers) and γ (trimers) could be performed in a fused-silica capillary filled with 4% non-cross-linked polyacrylamide (Fig. 3) [10]. Separations were run in a 50 mM Tris–glycine buffer at pH 8.8 with the sampling port at the anodic end of the capillary; samples (chains) were denaturated by SDS. This method offers the possibility of separating off chain polymers of relative molecular mass 300,000 and higher. This is of considerable importance, because no method offering sufficient sensitivity is available for analysing such polymers (as a matter of fact, in most cases these emerge as a single broad peak). In diluted slab gels these can be separated; chromatographic methods do not offer sufficient selectivity.

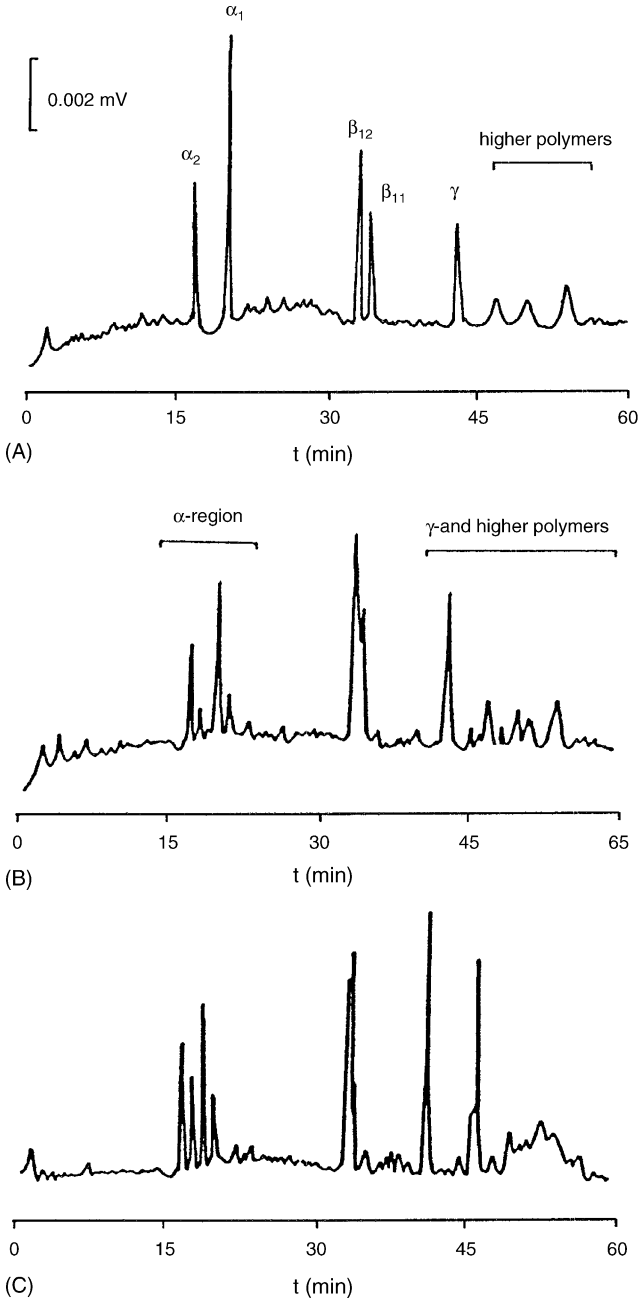

Fig. 3. Capillary gel electrophoresis profiles of collagen chains (4% polyacrylamide, Tris–glycine buffer pH 8.8). (A) Untreated collagen, (B) after incubation with glucose for 4 days, and (C) 7 days. Reprinted from Deyl and Miksik [10], with permission from Elsevier.

From a practical point of view, this method could be applicable for research into the posttranslational modification of collagen. It has been demonstrated on the study of glycation (the formation of crosslinks by reaction with sugar). This reaction produced a splitting of both α-peaks into two (i.e. four peaks for the α-chains), and in an increase in the number and intensity of the peaks for the γ-chain polymers and higher (Fig. 3). Both of these effects (splitting of α-chains and increasing of polymers) can be ascribed to the interaction of free lysine amino groups with glucose. It has been shown by Reiser et al. [11] that such interactions are non-specific, affecting several lysine residues in the collagen molecule. It could be assumed that the splitting of the two α-chain peaks into four upon incubation may reflect the monotopical binding of glucose to several sites along the collagen α-chain and the higher proportion of γ- and higher α-chain polymers could be explained on the basis of glucose-polymerising reactions, which eventually lead to the decreased solubility of the treated collagen samples.

Another approach for the analysis of bovine collagens used by Chang et al. [12]. Instead of linear polyacrylamide gel they used the commercially available Bio-Rad CE-SDS kit. The CE-SDS Protein Kit is characterized by the manufacturer as a polymer solution for creating a dynamic sieving effect [13]. This kit could be suitable for the separation of proteins over a broad molecular mass range (14–200 kDa) and for the determination of their molecular mass. The authors optimized many parameters of separation (capillary length, internal diameter, voltage) and injection (injection mode, time). The optimized method utilized a 36 cm (or 24 cm) × 50 μm uncoated capillary, electrophoretic injection at 10 kV for 10 s, a run voltage of 15 kV, a capillary temperature of 20 °C, and UV detection at 220 nm. The separation was the same for uncoated and polyvinylalcohol-coated capillaries. It is worth mentioning that the commercially available kit (buffer) has a relatively high pH (ca. 8.9). The assay had good repeatability (R.S.D.s for the peaks were 1–5%), enables us to quantitate the chains and the responses were linear for assay solutions with collagen concentrations from 0.125 to 1.25 mg/ml. The DSCE electropherogram of bovine skin collagen provided a profile of chains (α, β and γ) similar in number and relative abundance to that generated by the scanning of Coomassie-stained SDS-PAGE gels (Fig. 4).

Collagen methylation was studied using capillary electrophoresis in a polyvinyl alcohol-coated fused-silica capillary with a phosphate buffer (50 mM, pH 2.5) with 0.05% hydroxylpropylmethylcellulose (HPMC) [14]. The collagen used was mainly collagen type I (95–98%, the rest was collagen type III). Capillary zone electrophoresis was used in principle, but with a gel additive. The concentration of HPMC has an influence on the separation—an increase in concentration leads to the deterioration of resolution between the methylated and un-methylated collagens. The authors interpreted this phenomena as probably caused by better sieving effects in the resolution of proteins

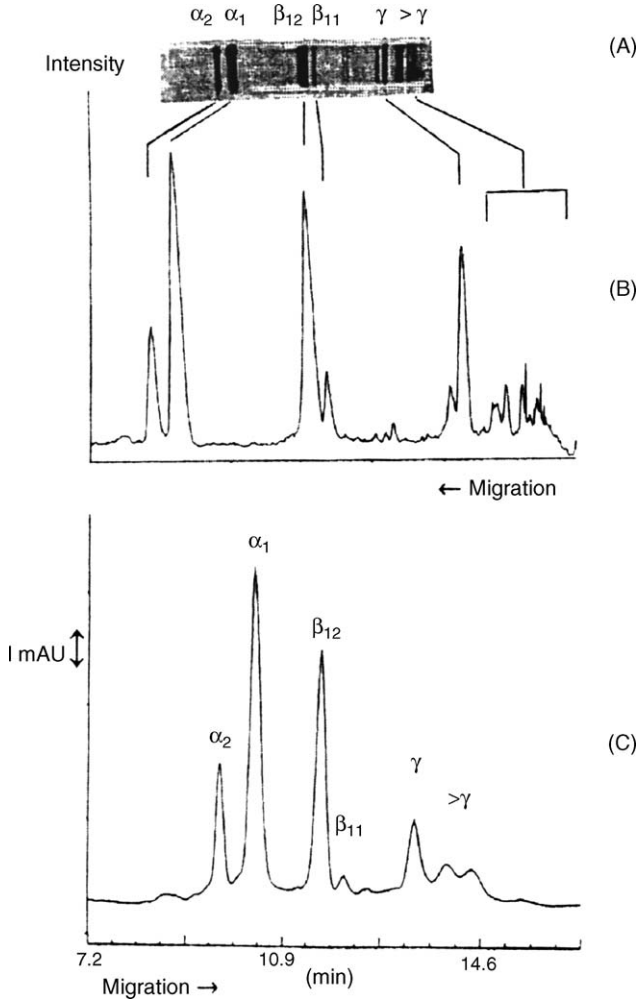

Fig. 4. Dynamic sieving capillary electrophoresis (DSCE) of bovine skin collagen. (A) Slab gel electrophoresis (SDS-PAGE), (B) optical density of SDS-PAGE, and (C) DSCE. Reprinted from Chang et al. [12], with permission from Elsevier.

possessing high molecular mass. This effect will probably be mainly useful for identifying small changes in the structure of long proteins. The system used was satisfactory for the determination of methylated collagens into four major bands that changed with different methylation reaction conditions.

3. CNBr fragments

Analyses of the structure and modifications to the collagen molecule/chain require solubilization and fragmentation of the protein to smaller peptides. In principle, two methods can be used—nonenzymatic (chemical) or enzymatic treatment. The chemical method is cleavage by bromcyan (CNBr). CNBr splits the protein molecule at specific locations—at the methionines (in this case towards the C-terminal end). In the collagen molecule, methionine is a relatively rare amino acid (some 10–20 amino acids per collagen molecule). The small number of methionine residues leads to a rather limited number of cleavage products (CNBr peptides). The profile of CNBr peptides is typical, at least for the main collagen types, and thus provides an appropriate way to estimate the amount as well as type of collagen in a particular tissue [15]. At present, there are several methods in use for CNBr peptide analysis, with classical CM cellulose [16] and phosphocellulose [17] chromatography being those primarily mentioned. The disadvantage of ion-exchange chromatographic procedures is mainly due to their low selectivity, long analysis time and poor recovery of the separated peptides. In the early 1980s Smolenski et al. [18] and van der Rest et al. [19] introduced reversed-phase chromatographic procedures, which exhibited much higher selectivity and shorter analysis time. However, the most widely-used method for CNBr peptides analysis today is gel electrophoretic separation, originally introduced by Scott and Veis [20].

The nomenclature of CNBr peptides refers to the parent polypeptide chain: an α_1 polypeptide chain yields a set of α_1CB (i.e. α_1CNBr) peptides (an α_2 chain similarly yields a set of α_2CB peptides). The index, e.g. α_1CB_1, identifies a particular peptide within the set. The number in parenthesis refers to the collagen type, e.g. α_1(I)CB_1 means a CNBr peptide of collagen type I. 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.

All of the peptides are present only once in the individual parent polypeptide chains: this means that the molar ratio of any CNBr peptide arising from an α_1 chain to any CNBr peptide arising from the α_2 chain in type I collagen must be 2:1 (because there are two α_1 chains and one α_2 chain per collagen type I molecule). The range of peptide molecular masses is broad, from less then 5000 (e.g. Mw 3300 for α_1(I)CB_2 and α_1(I)CB_3) to 29,000 (α_2(I)CB_4) or even 60,000 (α_2(I)$CB_{3,5}$—resulting from incomplete cleavage).

3.1. Capillary zone electrophoresis

CNBr fragments strongly adhere to the fused-silica capillary wall. For this reason, the separation of these peptides can only be realized in an acidic buffer.

The separation of the higher molecular mass peptides (over 13,500) from a CNBr fragmentation of collagens types I and III exhibits a linear relationship between mobility and molecular mass [21] when a phosphate buffer is used as the background electrolyte, and a rich spectrum of peptides can be obtained. The method is flexible—conditions can vary over relatively broad ranges: pH from 2.0 to 2.5, buffer concentration between 25 and 100 mM, applied voltage 8–25 kV (for a capillary of 70/63 cm total length/length to the detector, 75 μm i.d.). It is proposed that the interaction between the peptides and capillary wall is multimodal (involving more than one interaction) because using a coated (modified) capillary ruins the analysis, as does using an organic solvent [21]. On the other hand, the separation resembles reversed-phase HPLC and separation may involve hydrophobic sorption to the capillary wall [22]. Due to the structural rigidity (repeating of typical tripeptide sequence) of typical high-molecular CNBr fragments of collagen, the hydrophobic properties of these peptides are similar and so probably this is why their migration rates resemble their molecular masses. As was mentioned earlier, the increase in migration time with increasing

molecular mass holds for peptides with a relative molecular mass of 13,500 (149 amino acids) or more. Peptides that have a relative molecular mass of less than 4600 did not follow this relationship between relative molecular mass and migration time. This method is also suitable for the determination of higher molecular mass peptides arising from the posttranslational modification of proteins (cross-links due to glycation reactions). The same relationships were demonstrated on the separation of CNBr peptides including low molecular mass peptides from collagens type III (α_1(III)CB_2) and V (α_1(V)CB_1) [23]. The separation of a mixture of these collagen fragments (including spiked markers of collagen types III and V) is demonstrated in Fig. 5. It must be noted that a BioRad pH 2.5 buffer was used in this separation. This buffer contains a polymeric (molecular sieving) additive, probably hydroxymethyl cellulose. The separation in a buffer with an additive is similar to that in a plain phosphate buffer, the only observable difference is in the loss of the largest peptide, incompletely cleaved α_2(I)$CB_{3,5}$, and more stretching of the peaks in the region of higher molecular mass peptides.

A collagen type's specific low molecular mass peptides can be used to determine the proportion of a given collagen types (I, III and V). For this purpose three peptides were selected—α_2(I)CB_4, α_1(III)CB_2 and α_1(V)CB_1. The separation of type I collagen fragments spiked with marker peptides can be seen in Fig. 5. The relative determination of collagen types is based on the peak areas of selected peaks (markers). It was demonstrated that this method is suitable for determining the proportions of these three collagen types present in tissues.

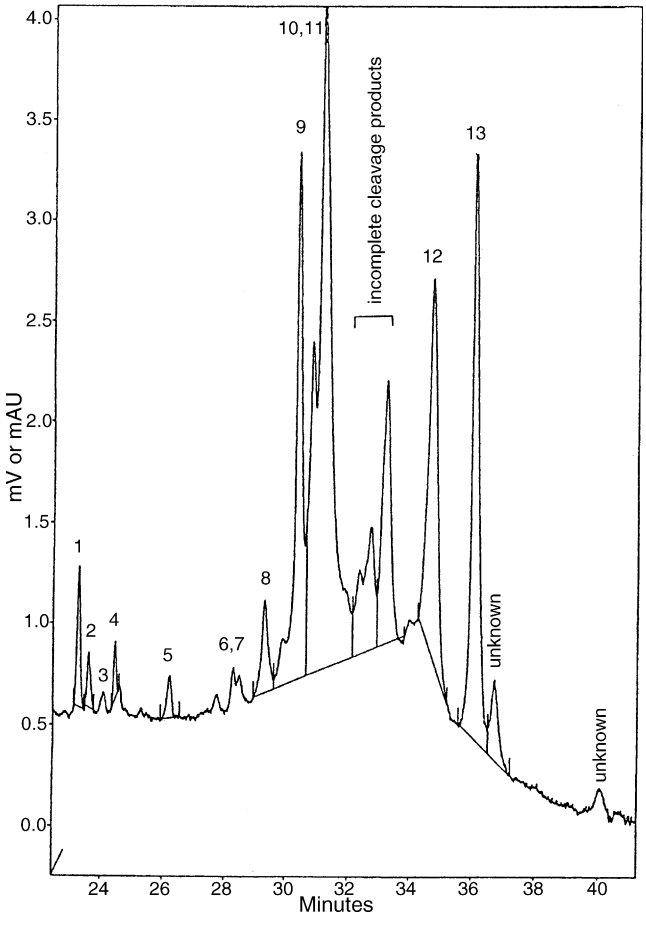

Fig. 5. Separation of collagen type I CNBr peptides obtained from commercial preparation (Sigma) spiked with α_1(V)CB_1 and α_1(III)CB_2. Background electrolyte Bio-Rad pH 2.5 phosphate buffer (with polymeric modifier) diluted 1:1 with Milli-Q water. Peak identification: (1) α_1(I)CB_2, (2) α_1(I)CB_4, (3) α_1(V)CB_1, (4) α_2(I)CB_1, (5) α_1(III)CB_2, (6) α_1(I)CB_5, (7) α_2(I)CB_2, (8) α_1(I)CB_3, (9) α_1(I)CB_6, (10) α_1(I)CB_7, (11) α_1(I)CB_8, (12) α_2(I)CB_4, (13) α_2(I)$CB_{3,5}$. Reprinted from Deyl et al. [23], with permission from Elsevier.

Cyanogen bromide fragments of collagen were also used for the analysis of peptides specific to types I and II in the cartilage [24]. The authors of this study evaluated a broad range of conditions of separation and found that the best conditions, that enable the separation of type-specific peptides, were separation in a Supelco CElect N capillary using a 100 mM phosphate buffer at pH 6. They used this method for the characterization of meniscal cartilage. It should be mentioned that the CElect N capillary is a product of Supelco (Sigam-Aldrich) and it is based on a two-stage method (treating the capillary surface with a vinyl group containing silane or polysiloxane, then copolymerizing a hydrophilic monomer onto the vinyl/silane layer in situ) for preparing stable and hydrophilic CE column coatings that show virtually no electroosmotic flow from pH 2 to 10 [25].

The separation of lower-molecular CNBr peptides can be improved by adding an ion-pairing agent, heptanesulfonic acid [26]. The separation reflected the ion-pairing equilibria between the ion-pairing agent and the peptide/protein analytes. The influence of ion-pairing on sample mobility (running time) was more pronounced with higher-molecular peptides compared to lower-molecular ones. This different influence offers the possibility to separate low and high molecular CNBr peptides that would co-migrate in the absence of the ion-pairing agent, as was demonstrated on the collagen of rat tail tendon. The influence of heptanesulfonic acid on the separation of low-molecular mass peptides (after the elimination of higher-molecular mass peptides by ulrafiltration) is shown in Fig. 6.

3.2. *Micellar electrokinetic chromatography and capillary gel electrophoresis*

Another possibility for minimizing the interaction of proteins with the capillary wall is using a high concentration of surfactant (above the critical micellar concentration) that is, micellar electrokinetic chromatography (MEKC). This was demonstrated with the use of SDS [27]. Separation was made in a system consisting of a 50 mM phosphate buffer (pH 2.5) with 50 mM SDS. This system is suitable for the separation of a peptide mixture, where in some cases plate counts of 100,000 can be achieved. It should be highlighted that the peptide-micelle associates move rapidly to the anode. At acidic pH electroosmotic flow is low (if any), and so at this pH the system has to be run in negative polarity mode. At low, submicellar concentrations the separations are different and reflect only interactions between the peptides and with the capillary wall but not the presence of SDS micelles in the background electrolyte. In the presence of SDS, the separation differs substantially [28]. Capillary electrophoresis can be carried out either in a 25 mM phosphate buffer (pH 2.5) or in a 25 mM phosphate buffer (pH 4.5) diluted to 0.1% with respect to SDS. While in the first case (pH 2.5) the peptides move to

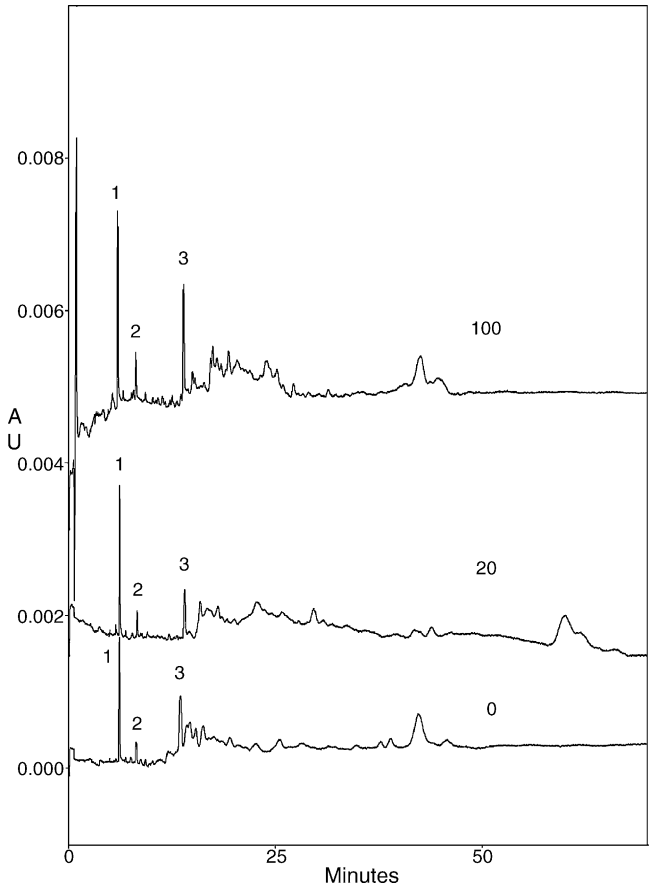

Fig. 6. Electrophoregrams of low-molecular weight fraction (below 10×10^3) of CNBr collagen fragments (rat tail tendon collagen). Separations made in 50 mM phosphate buffer, pH 2.5 only (0), 50 mM phosphate buffer, 20 mM heptanesulfonic acid, pH 2.5 (20) and 50 mM phosphate buffer, 100 mM heptanesulfonic acid, pH 2.5 (100). Reprinted from Miksik et al. [26], with permission from Elsevier.

the cathode in a molecular mass-dependent manner, in the second (pH 4.5 with SDS) they move towards the anode (also in a molecular mass-dependent manner).

Another interesting possibility is the use of block copolymers. These copolymers can self-assemble to form micelle structures in aqueous buffers. This property makes these materials highly appealing for capillary gel electrophoresis and they are another alternative to linear polymeric materials. Especially interesting copolymers are thermoadjustable-viscosity polymers. Typical examples of this category are Pluronic polymers. Pluronic polymers are triblock uncharged copolymers with the general formula $[\text{poly(ethylene oxide)}]_x[\text{poly(propylene oxide)}]_y[\text{poly(ethylene oxide)}]_x$ (Pluronic is a registered trade name of BASF Performance Chemicals, Mount Olive, NJ, USA). Of these types of copolymers, Pluronic F127 is often used (with coefficient values of roughly $x = 100$ and $y = 70$ and a molecular mass of about 13,000). These copolymers have the typical features of surfactants and self associate into large micelles. Self-association is favored by increasing concentration and temperature. The less polar poly(propylene oxide) chain segments are desolvated and segregate into a hydrophobic micelle core surrounded by a soft "brush" of highly hydrated, flexible poly(ethylene oxide) chains. Pluronic copolymers form both isotropic and anisotropic liquid crystalline "gels". The type of phase (isotropic, cubic, hexagonal or lamellar) depends not only on the structural features of the polymer but also on its concentration and temperature. This means in practice that a Pluronic which is soluble at low temperature can gellify with a temperature increase, e.g. Pluronic F127 at a concentration of 20% is a freely flowing liquid at refrigerator temperature (5 °C). At this stage, the polymer can be easily introduced into the capillaries. At room temperature (20 °C) this liquid forms a gel [29,30].

Pluronic media can be used for the separation of CNBr collagen peptides, where 7.5% Pluronic F127 (in a pH 2.5, 10 mM Tris and 75 mM phosphate buffer) was used [31]. The separation was significantly improved in comparison with the separation achieved in the buffer alone (without the Pluronic). The temperature used was 20 °C, a higher temperature (50 °C) influenced migration time but not resolution or migration order. The use of a higher concentration of gel caused too long a migration time and produced many "bumps" on the baseline. The separation resembles the separation made by reversed-phase HPLC, but with a different elution order as is obvious from Fig. 7 [31]. It is another confirmation of the proposal that CE and HPLC methods are not competitive but complementary. Pluronic media was validated as suitable media for the separation of peptides when it is assumed that the separation of proteins/peptides in the presence of Pluronic in the background electrolyte occurs on a charge/mass ratio basis with molecular sieving effects acting as a secondary partition mechanism [32]. The CNBr profile of collagen tissue was then subjected to analysis by the programme PeakFit which revealed 17 peaks: 15 sharp peaks and two broad ones (Fig. 8).

4. Collagenase fragments

Collagens can be cleaved by specific enzymes called collagenases. These enzymes are metalloendopeptidases. There are two major types of collagenases—tissue (interstitial) and bacterial [33]. Interstitial collagenase (EC 3.4.24.7) cleaves the triple helix of collagen at about three-quarters of the length of the molecule from the N-terminus (at 775Gly/Ile776 in the α-1(I)chain). Microbial collagenase, typically from *C. histolyticum* (EC 3.4.24.3), digests native collagen in the triple helix region at the Gly-bonds where preference was shown for Gly at P3 and P1'; Pro and Ala at P2 and P2'; and hydroxyproline, Ala or Arg at P3'. There are also other known types of collagenases, e.g. gelatinase B (EC 3.4.24.35) which cleaves gelatine type I and collagen types IV and V, gelatinase A (EC 3.4.24.24) which cleaves gelatine type I and collagen types IV, V, VII and X, neutrophil collagenase (EC 3.4.24.34) which cleaves interstitial collagens with a preference for collagen type I, and some other enzymes that are not well known but possess some collagenase activity (ECs 3.4.21.32, 3.4.24.17, 3.4.24.8, 3.4.24.9, 3.4.24.B2, 3.4.24.B4 and 3.4.24.B6).

4.1. Capillary zone electrophoresis

Deep cleavage of the parent collagen molecules can be performed using *C. histolyticum* collagenase (see above). This

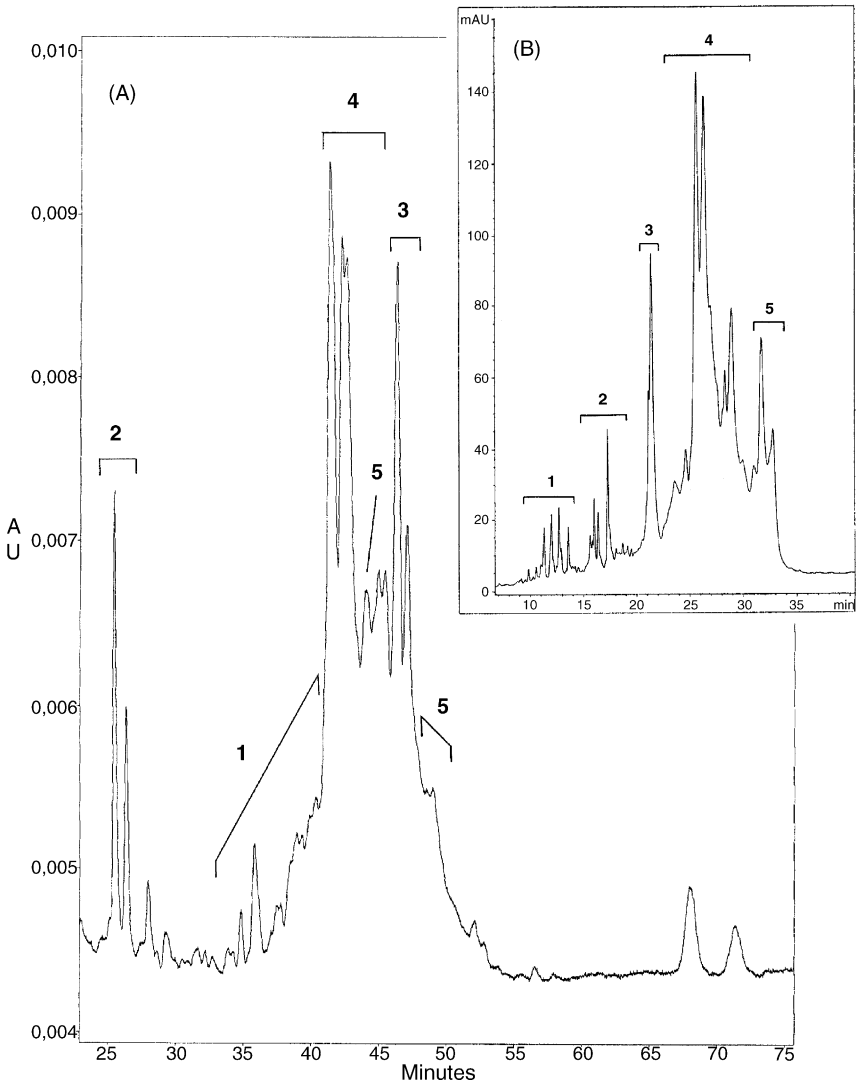

Fig. 7. Separation of rat tail tendon collagen CNBr peptides by (A) capillary electrophoresis in Pluronic copolymer media (10 mM Tris and 75 mM phosphate buffer, pH 2.5, containing 7.5% Pluronic F127 copolymer) and by (B) reversed-phase high-performance liquid chromatography. Identification of individual fractions: (1) α_2(I)CB$_2$, α_1(I)CB$_2$, α_1(I)CB$_5$; (2) α_1(I)CB$_4$; α_1(III)CB$_3$, α_1(III)CB$_6$, α_1(I)CB$_3$, α_1(III)CB$_4$; (3) α_1(I)CB$_6$; (4) α_1(III)CB$_5$, α_1(I)CB$_7$, α_1(I)CB$_8$, α_2(I)CB$_4$ and incomplete cleavage products; (5) α_2(I)CB$_{3,5}$, (α_1(III)CB$_9$)$_3$. Reprinted from Miksík and Deyl [31], with permission from Elsevier.

protease produces a very complex peptide mixture (theoretically about 172 peptides could arise from a naturally-occurring collagen types I and III mixture). The separation of these peptides (preferably tripeptides) is not a simple matter. It was demonstrated that short peptides with proline in the carboxy terminus adhere to the capillary wall [8]. For this reason a study was conducted of possible ways of modifying the silanophilic activity of the inner surface of the capillary wall by dynamic coating (alkylamines added to the background electrolytes) at acidic pH. The influence of these modifications was tested on a seven-membered test mixture of peptides typical of collagenase digest. From a set of eight amines (alkyl-α, ω-diamines, polymeric amines and cationic amine surfactants), the best results were obtained with 1,2-diaminoethane and 1,7-diaminoheptane. On the other hand, for the best separations for the slowly moving peaks were obtained with 1,7-diaminoheptane, hexadecyltrimethylammonium bromide and hexamethonium bromide, i.e. with modifiers possessing large aliphatic domains which are likely to be hydrophobically bonded with the separated solutes. The selectivity improvement with fast moving members of the test mixture could be ascribed to a decrease in electroosmotic flow, while the improved separation with slowly moving peaks appears to reflect the altered interaction with the hydrophobized

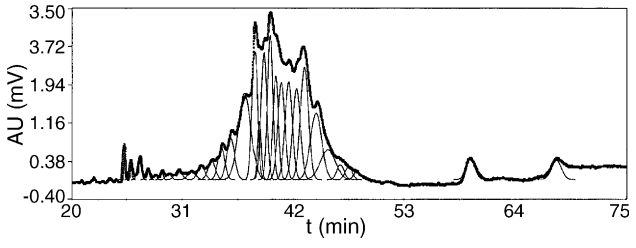

Fig. 8. Analysis of electrophoretic profiles by programme PeakFit. Separation with CElect P150 capillary 27 cm (20 cm to the detector) 350 μm i.d., 10 mmol/l Tris and 75 mmol/l phosphate buffer containing 7.5% (v/v) Pluronic F127, pH 2.9, 20 °C, 5 kV. Reprinted from Miksik et al. [32], with permission from Elsevier.

capillary wall. It was demonstrated that this modification can improve the separation of a peptide mixture arising from the bacterial collagenase treatment of a hydrolysate of collagen types I and III [34].

Although the separation of these collagenase peptides is not complete, the application of suitable mathematical methods can improve the evaluation of the data obtained. Principal component analysis (PCA) is an excellent method for the analysis of these profiles. An example of capillary electrophoretic separation at acidic pH (100 mM phosphate buffer, pH 2.5) used for PCA is shown in Fig. 9. Principle component analysis enables us to identify changes in the peptide profiles [35].

Capillary electromigration methods can be also successfully used for the determination of 1/4 and 3/4 fragments of collagens types I and II arising from cleavage by interstitial collagenase. Sano et al. [36] determined the collagenase activity by capillary gel electrophoresis (CGE) with laser-induced fluorescence detection. The activity of three collagenases against collagens types I and II were measured by determining the 3/4 fragments. High sensitivity was achieved by using a dynamic fluorescence labelling technique with a running buffer containing 0.05% sodium dodecylsulfate and a dye for protein, NanoOrange. NanoOrange is a fluorescein-like dye and it is non-covalently binding to the protein—SDS complex surface or hydrophobic domain of non-denatured proteins; it enhances the fluorescence sensitivity of protein due to conformational changes induced by binding. NanoOrange did not notably affect the protein—SDS complex mobility throughout the CGE separation [36]. The collagen (type I or II) and both fragments could be separated and detected within a run time of 20 min by CGE using a gel buffer (pH 8.8) containing 4% polyacrylamide. The buffer used was 50 mM AMPD-CACO (2-amino-2-methyl-1,3-propanediol–cacodylic acid) and the capillary was an eCAP-coated capillary (Beckman Coulter). The laser used was an argon ion 488 nm laser (Beckman). The assay of the 3/4 fragment was reproducible and was usable for examining the metalloproteinase inhibitor matrix.

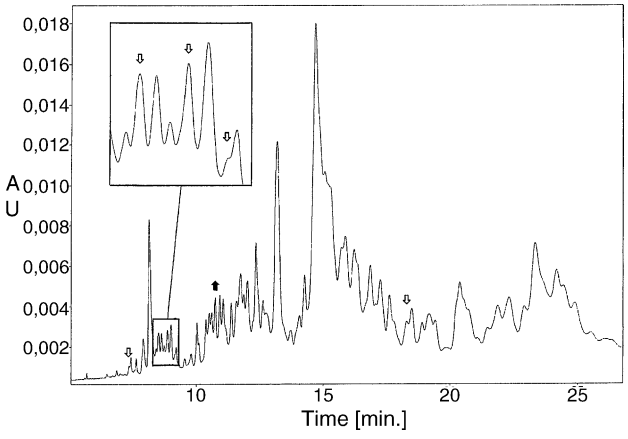

Fig. 9. CZE—peptide map obtained from tails of genetically hypertriglyceridemic rats after bacterial collagenase treatment. Increased and decreased peaks in comparison to control rats indicated by filled and unfilled arrows, respectively. Reprinted from Miksik et al. [35], with permission from Elsevier.

4.2. Combination of HPLC and capillary electrophoresis

As was mentioned above, the cleavage of tissue consisting of collagen types I and III by bacterial collagenase could theoretically result in a mixture of some 172 peptides. The separation of this peptide set by only one analytical method was unsuccessful—with RP-HPLC only 45 peaks could be determined and only 65 peptides with CE. This resolution is not sufficient for the study of collagens and their posttranslational modification. The above-mentioned methods were combined off-line and the number of peaks (and resolution) dramatically increased—150 peaks were detected. In the first stage, the peptide mixture was separated by reversed-phase HPLC (in a Zorbax Eclipse XDB C18 column, Agilent) using gradient elution with a water–acetonitrile system with trifluoroacetic acid as ion-pair agent. The collagenous peptides were divided into five fractions by HPLC. These fractions were further characterized by CE in an uncoated capillary using a phosphate buffer (100 mM, pH 2.5) (Fig. 10). This two-stepped peptide mapping with subsequent statistical evaluation (linear regression analysis) was shown to represent a reliable approach for revealing posttranslational modifications in collagen in vivo [37]. It should be highlighted

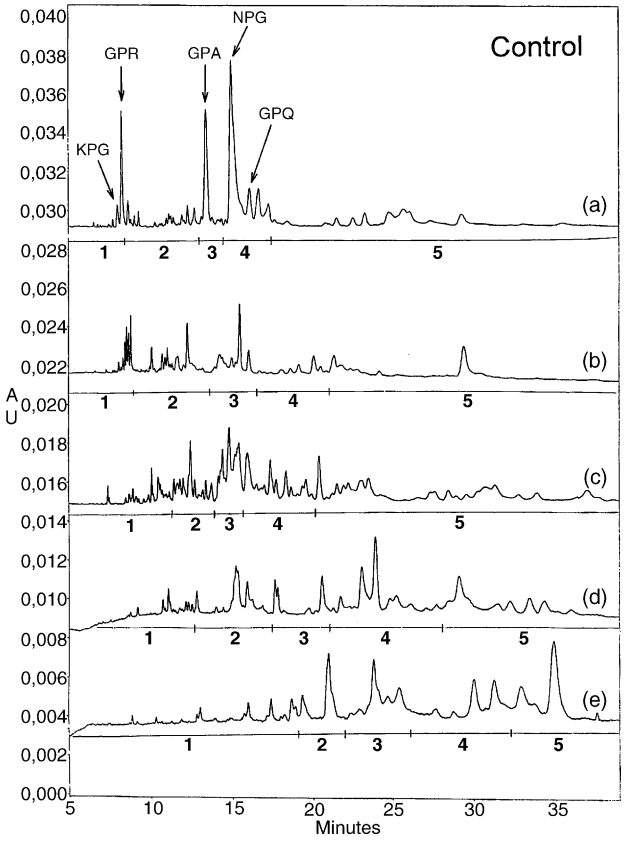

Fig. 10. Combination of HPLC and CE. Capillary electrophoretic profiles of individual fractions accumulated from HPLC runs. Numbers of individual recordings (1–5) refer to HPLC fractions. Further subsectioning of the electrophoretic profiles is clearly indicated in the Figures (this subsectioning was used for subsequent statistical analysis). Five typically collagenous peptides were identified in the first chromatographic section (a) as indicated. Reproduced from Eckhardt et al. [37], with permission from Taylor & Francis Group, LLC., http://www.taylorandfrancis.com.

that collagens, as slowly metabolised proteins, are candidates for a high extent of non-enzymic posttranslational modifications. The typical example is glycation, reaction between the oxo-group of a reducing sugar and free amino group of the protein. These modifications have now been extensively studied.

4.3. Coupling of capillary zone electrophoresis and MS

The coupling of capillary electrophoresis and mass spectrometry for peptide/protein analysis is a promising technique which will have a big impact on protein research [38]. Surprisingly, this method has not yet been applied in the analysis of collagen.

Our group developed a method for analysing collagenase (from *C. histolyticum*) digests of collagens. The separation of collagen peptides was carried out in a fused-silica capillary (100 cm × 75 μm i.d.) with a background electrolyte consisting of 0.25 M acetic acid, at an applied voltage of 20 kV. The instrument used was a Beckman P/ACE 5000 and it was coupled to a mass spectrometer made by Hewlett–Packard (now Agilent; quadrupole MSD 1100). These instruments were coupled with a grounded needle carrying a flow of sheath liquid. The sheath liquid used was 5 mM ammonium acetate/isopropanol 1:1 (v/v) at a flow-rate of 6 μl/min. The mass spectrometry (atmospheric pressure ionization–electrospray ionization at positive polarity) conditions were: drying gas (N_2) 8 l/min; drying gas temperature 150 °C; nebulizer pressure 5 psi; capillary voltage 3500 V; ions were observed over a mass range of *m/z* 100–1500. The fragmentor was set at 100 V. This method enables us to identify typical collagenous tripeptides in the digest (see Fig. 11).

5. Collagenous amino acids and cross-links

Capillary electromigration methods are also used for the determination of a typical collagenous amino acid–hydroxyproline. Vallejo-Cordoba et al. [39] performed an overview of capillary electrophoretic methods for the determination of hydroxyproline as a specific marker for collagen determination in meat and the analysis of meat quality. In principle, we could say that hydroxyproline can be separated the same way as other amino acids. The main difference lay in the structure of proline-based amino acids—they are secondary amino acids.

Proline and hydroxyproline levels can be determined after derivatization by fluorescamine and subsequent analysis by capillary electrophoresis. Fluorescamine reacts readily with secondary amino acids to form nonfluorescent aminoenone-type chromophores which are easily detected in the low UV region [40].

An interesting possibility is to utilize another secondary amino acid reaction for selective determination of proline-based amino acids. For example, the derivatization of hydroxyprolines (3- and 4-hydroxyproline) by phenyl isothiocyanate could be used for their quantification [41]. Phenylthiohydantoin (PTH) derivatives of 3- and 4-hydroxyproline (Hyp) were separated using (MEKC). Amino acids from hydrolyzed tissues were labelled using a two-step procedure that involved initial reaction with *o*-phthalaldehyde (OPA) to modify the primary amines followed by their precipitation under acidic conditions. In the second step, amino acids with a secondary amino group were reacted with phenyl isothiocyanate (PITC). A separation protocol was also used to determine the Hyp content of bovine skeletal perimysial collagen preparations and whole muscle samples.

Another method uses a similar principle, i.e. derivatization by OPA in the first stage to reduce primary amine interference and then derivatization of hydroxyproline by another derivatization reagent, in this case 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD) [42]. The derivatives that arose were analysed using micellar electrokinetic chromatography with

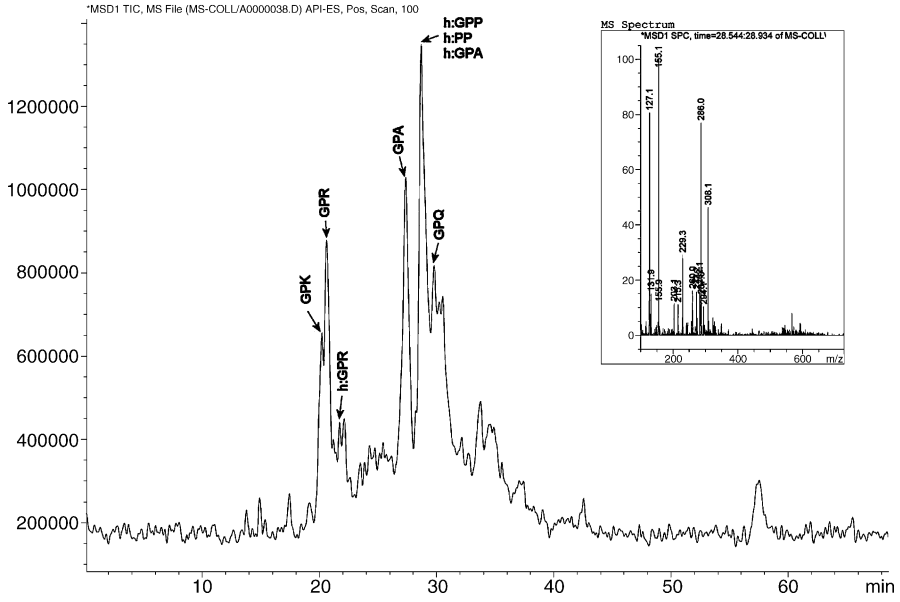

Fig. 11. Coupling of CZE and MS. Analysis of rat skin collagenase digest (microbial collagenase from *Clostridium histolyticum*) by coupling capillary zone electrophoresis and mass spectrometry (for details see text). *Y*-axis: total ion current. Identification: see figure, h indicates hydroxylation of proline. Inset: mass spectrum of peak at migration time 28.544–28.934 min.

laser-induced fluorescence detection. This method was used for the quantification of 4-hydroxyproline in muscle hydrolysates when pyrrolidinol was used as an internal standard.

Kaml et al. [43] described a different approach—the determination of underivatized amino acids by capillary zone electrophoresis. Separation was made at acidic pH (2.26) and a conductivity detector was used for detection. This method allows us to identify different species of proteinaceous binders—collagen, egg white, and milk casein by using the following markers: hydroxyproline, proline, glycine, glutamic acid, serine and valine. The combination of the mentioned markers served for identification of proteins: higher content of hydroxyproline and glycine (hPro > 1% and gly > 15%) allows to distinguish collagen from egg white and caseine; content of praline, glutamic acid and serine + valine allows to distinguish egg white and caseine (pro > 8%, glu > 20%, ser + val < 18% indicate caseine). Other interesting collagenous substances are crosslinks. Veraat et al. [44] developed a capillary electrophoretic method for the determination of typical collagen crosslinks hydroxylysylpyridinoline (HP), and lysylpyridinoline (LP). This method uses a frequency-doubled Rhodamine dye laser at excitation frequencies of 290 and 325 nm. The emission was measured with an intensified diode-array detector mounted on a spectrograph to obtain wavelength-resolved spectra. The only way to achieve acceptable concentration detection limits was by using LIF detection, i.e. 200 nM HP and LP in a 30 mM phosphate buffer (pH 2.0).

6. Conclusions

Collagens are highly important proteins found in many locations in animal anatomy. We can assume that the analysis of these proteins (of their constitution and modification during aging, physiological and pathophysiological changes) will be intensified in the future. This assumption is in agreement with the contemporary era of proteomic research, with an era of analysis and explanation of the role of proteins in life at various levels of research. Because capillary electromigration techniques are one of the most effective analytical methods, it should be assumed that they will play an important role in the research of collagen. We currently have a broad spectrum of these methods at our disposal, but it should be presumed that during the proposed increasing interest in collagen analysis, new methods will be developed.

Acknowledgements

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