Collagen: HPLC and Capillary Electromigration Methods

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Abstract
Collagen is a broad group of the most abundant proteins in the human body that encompasses at least 28 known types in vertebrates. These proteins are poorly soluble (if at all) and, therefore, they are often split into shorter peptides by enzymatic or nonenzymatic methods before analysis. Collagenous peptides can be successfully separated by high-performance analytical methods—high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). The HPLC methods use, most frequently, reversed-phase columns with ion-pairing. Various types of capillary electromigration methods can be used for the separation of collagens and their fragments. The main problem with capillary electrophoretic methods is adhesion of collagenous peptides to the surface of a fused-silica capillary. It can be eliminated by the use of acidic background electrolytes and/or use of surfactants in the separation medium. The high performance method for analysis is combination of HPLC and CE. Nowadays, the high-performance approach is the combination of HPLC and CE with mass spectrometry (MS).

INTRODUCTION
The term collagen covers a broad group of proteins. It is a family of extracellular matrix proteins possessing 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-. The protein chains are coiled together into a left-handed helix and are then wound around a common axis to form a triple helix with a shallow right-handed superhelical pitch; 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. 4-Hydroxyproline plays particularly an important role, because these residues are essential for the stability of the triple helix. All collagens also have noncollagenous domains.

Collagens are the most abundant proteins in the human body, constituting approximately 30% of its protein mass. At present, there are at least 28 known collagen types in vertebrates, containing a total of 42 distinct α-chains, and more than 20 additional proteins have collagen-like domains. Besides α-chains, there are also β-chains (dimers of α-chains) and γ-chains (trimers of α-chains).

The most common types of collagens occur in fibers 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 non-enzymatic posttranslational modifications.

Polymerized fibril-forming collagens (whether polymerized physiologically or nonphysiologically) are insoluble and their solubilization is routinely performed either by mild pepsinization, in which short terminal regions containing the polymerization sites (crosslinks) are cleaved off, or by cyanogen bromide (CNBr) cleavage, which results in a limited fragmentation of the parent α-chains, as mentioned previously. Tissue collagenases 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 into small fragments (mostly tripeptides) and are, therefore, of little use in structural studies.

Investigations of these proteins can either 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 (HPLC), low-pressure and high-performance liquid chromatography, but recently capillary electromigration methods have also begun to be used.

STANDARD (LOW-PRESSURE) LIQUID CHROMATOGRAPHIC SEPARATION PROCEDURES

The application of classical (low-pressure) chromatography for the isolation of fibril-forming collagens from tissues has a long tradition and involves a large number of methods. Practically all types of chromatographic operational modes have been utilized for this purpose [for a review, see, e.g., Deyl and Adam (1989)] and frequently strategic combinations of them are used, exploiting,
e.g., the presence or absence of S–S bonds in the terminal region (or along the whole molecule as is the case with collagen type III), charge, molecular size, the presence of glycosidic residues, or differences in the physicochemical properties of individual collagen species in their native and denatured forms.

The most common methods for the preparation of collagens are various extractions and precipitations or the release of protein by enzymatic hydrolysis. These methods mainly include extraction with an NaCl–phosphate buffer, acetate buffer, or acetic acid, and precipitation using various concentrations of NaCl (their usage depends on the tissue and type of collagen).

The most useful low-pressure chromatographic method for the purification of collagens is diethylaminoethyl (DEAE)-cellulose chromatography for removing co-extracted proteoglycans. Typical conditions for this form of chromatography can be as follows: the sample of collagen is dissolved in 0.2 mol/L NaCl with 0.05 mol/L Tris–HCl at pH 7.5 and fed into a DEAE-cellulose column (e.g., Whatman DE-52) which was equilibrated with the same buffer. After that, collagens are eluted with additional buffer, and proteoglycans are eluted by an increasing concentration of NaCl (1 mol/L).

Other chromatographic methods should also be mentioned: gel-permeation methods (molecular sieving), carboxymethyl (CM) cellulose chromatography, bioaffinity chromatography (Concanavalin A, thiol-activated Sepharose, Heparin Sepharose) or zone precipitation chromatography. Cation-exchange chromatography and phosphocellulose chromatography are very popular methods (elution is carried out with an NaCl gradient).

The separation steps are most often monitored by SDS-PAGE.

HPLC AND CAPILLARY ELECTROPHORETIC METHODS

Separations of collagens must contend with the problems of achieving high resolution among poorly soluble high molecular mass proteins or a complex mixture of peptides with similar structures (glycine at every 3rd position). This task makes high demands on advanced high performance separation methods—HPLC and capillary electrophoresis (CE).

Parent Chains

HPLC

The analysis of collagenous chains is a difficult task due to the poor solubility (hydrophobicity) of these proteins. HPLC methods are not frequently used for the separation/characterization of individual chains of a collagen type.

The reversed-phase HPLC method is a traditional method for the analysis of peptides and proteins. A good choice for the stationary phase could be a short-alkyl reversed-phase (e.g., C₄) with wide pores (30 nm). It has been shown that large pore supports give distorted peaks with small collagens and triple helical peptides, resulting in poor resolution. The formation of broad peaks has been ascribed to conformational instability of the separated solutes and slow cis–trans isomerisation of the peptide bonds. The best sorbents of those examined were diphenyl or nonporous C₁₈ reversed-phases; standard water–acetone gradients were recommended as mobile phases.

For example, reversed-phase chromatography on a C₈ phase using an acetonitrile gradient in 0.1% trifluoroacetic acid was described as a suitable method for the separation of α-chains of collagen types V and XI.

Cyanopropyl bonded packing has been described as suitable for the separations of human type I–III collagens. Other stationary phases should also be mentioned: LiChrosorb Diol, TSK-SW gels and Separos HEMA 1000 Glc (a copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate covalently bonded with glucose). The first two phases are widely used for the separation of a number of proteins, but the use of the last phase (HEMA) is mainly used for the separation of collagens. The elution is performed by isocratic conditions with 0.2 M NaCl–2 M urea–0.05 M Tris/HCl (pH 7.5) as the mobile phase. This enabled the separation of α-, β-, and γ-chains of collagen type I and this method also enables the separation of a number of collagens and collagenous chains (the related molecular mass of the eluted proteins decreases with prolonged migration time).

Capillary electrophoresis

Collagen chains can be analyzed by capillary zone electrophoresis in very dilute buffers (typically 2.5 mM sodium borate, pH 9.2) or acidic buffers (pH ~2.5, about 25 mM buffer). Separation in an alkaline buffer is sensitive to overloading; the recommended buffer is sodium tetraborate. The reason for this sensitivity is probably due to the adsorption of protein onto the capillary wall.

The separation of groups of α, β, and γ can also be achieved in a phosphate buffer (at the same pH), but the resolution of individual chains (α₁ from α₂, etc.) was lost. A similar effect was observed if the background electrolyte contained a submicellar concentration of sodium dodecyl sulphate (SDS), when only the peaks of α, β, and γ fractions could be seen. At higher (supramicellar) concentrations of the negatively charged surfactant, all fractions migrated as a single broad zone. The separation of collagen chains under acidic conditions (100 mM phosphate buffer, pH 2.5) is possible, but again without separating tri-, di-, and monomers.

Another possibility is to use a capillary gel electrophoretic method that is nowadays a routinely and
commercially available method for the determination of the molecular mass of proteins/polypeptides. This method can also be used for the separation of collagen chains and their polymers. For example, this procedure is described in the literature for the separation of collagen type I \( \alpha \)-chains and chain polymers \( \beta \) (dimers), and \( \gamma \) (trimers), and also chain polymers of related molecular mass 300,000 and higher (typically in the study of the formation of crosslinks). Besides commercially available kits, another option is to use fused-silica or polyvinylalcohol-coated capillaries filled with noncrosslinked polyacrylamide or hydroxylpropylmethylcellulose in a 50 mM Tris–glycine buffer (pH 8.8) or phosphate buffer (50 mM, pH 2.5) (Table 1).

### CNBr Fragments

Analyses of the structure and modifications of 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 CNBr. Cyanogen bromide 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. The nomenclature of CNBr peptides refers to the parent polypeptide chain: an \( \alpha_1 \) polypeptide chain yields a set of \( \alpha_1\text{CB} \) (i.e., \( \alpha_1\text{CNBr} \)) peptides (an \( \alpha_2 \) chain similarly yields a set of \( \alpha_2\text{CB} \) peptides). The index, e.g., \( \alpha_1\text{CB}_1 \), identifies a particular peptide within the set. The number in parenthesis refers to the collagen type, e.g., \( \alpha_1(I)\text{CB}_1 \) 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.

### HPLC

At present, there are several methods in use for CNBr peptide analysis, with classical CM cellulose and phosphocellulose 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, reversed-phase chromatographic procedures were introduced, which exhibited much higher selectivity and shorter analysis time. The most useful method is based on a separation using a C18 reversed phase in an acetonitrile gradient (0–40% acetonitrile over 90 min) containing heptafluorobutyric acid as an ion-pairing agent. However, the most widely used method for CNBr peptides analysis today is gel electrophoretic separation, originally introduced in 1976.

### Capillary electrophoresis

The main problem in separating collagenous peptides is their adhesion to the inner surface of the fused-silica capillary wall. For this reason, the separation of these peptides can only be achieved in an acidic buffer. In the literature, the separation is carried out in a 25–100 mM phosphate buffer (pH from 2.0 to 2.5), applied

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**Table 1** Capillary electromigration methods for separation of collagens

<table>
<thead>
<tr>
<th>Collagen types</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha, \beta )-chains (types I, II, V, IX, XI)</td>
<td>2.5 mM Sodium tetraborate buffer, pH 9.2</td>
</tr>
<tr>
<td>( \alpha, \beta, \gamma )-chains (type I)</td>
<td>4% Non crosslinked polyacrylamide; 50 mM Tris–glycine buffer, pH 8.8</td>
</tr>
<tr>
<td>( \alpha, \beta, \gamma )-chains (bovine skin–type I)</td>
<td>Commercial CE-SDS kit (pH 8.9)</td>
</tr>
<tr>
<td>Cyanogen bromide (CNBr) fragments (rat tail tendon—types I and III)</td>
<td>25–100 mM Phosphate buffer, pH 2.0–2.5</td>
</tr>
<tr>
<td>CNBr fragments (rat tail tendon—types I and III)</td>
<td>100 mM Phosphate buffer, 20 mM heptanesulfonic acid, pH 2.5</td>
</tr>
<tr>
<td>CNBr fragments (cartilage—types I and II)</td>
<td>100 mM Phosphate buffer, pH 6; coated capillary</td>
</tr>
<tr>
<td>CNBr fragments (rat tail tendon—types I and III)</td>
<td>50 mM SDS in 50 mM phosphate buffer, pH 2.5</td>
</tr>
<tr>
<td>CNBr fragments (rat tail tendon—types I and III)</td>
<td>1% (cca 3.5 mM) SDS in 50 mM phosphate buffer, pH 2.5</td>
</tr>
</tbody>
</table>
| CNBr fragments (cartilage—types I and II)         | 7.5% Pluronic F127 in 10 mM Tris and 75 mM phosphate buffer, pH 2.5;
|                                                      | 100 mM Phosphate buffer, pH 2.5                                  |
| Bacterial collagenase fragments (rat tail tendon—types I and III) | 100 mM Phosphate buffer, pH 2.5                                  |
voltage 8–25 kV (using a fused silica capillary of 70/63 cm total length/length to the detector, 75 µm i.d.). Peptides with higher related molecular mass (over 13,500) from collagens types I and III demonstrate a linear relationship between mobility and molecular mass; however, peptides with lower related molecular mass (below 4600) do not follow this relationship. This method is also usable for the determination of collagen types I, III, and V based on their specific CNBr fragments (z2(I)CB3, z1(III)CB2, and z1(V)CB1). The separation of lowermolecular CNBr peptides from the higher-molecular ones can be improved by adding an ion-pairing agent, heptanesulfonic acid (100 mM) to the separation buffer.

Another possible way to minimize the interaction of collagen with the capillary wall is the presence of a high concentration of surfactant (above the critical micellar concentration), i.e., by micellar electrokinetic chromatography (MEKC). A useful system consists of a 50 mM phosphate buffer (pH 2.5) with 50 mM SDS; this system has to be run in negative polarity mode. At low, submicellar concentrations, the separations are different and only reflect interactions between the peptides and with the capillary wall, but not the presence of SDS micelles in the background electrolyte.

A pluronic polymer, which is a triblock uncharged copolymer with the general formula [poly(ethylene oxide)]n[poly(propylene oxide)]m[poly(ethylene oxide)]n, was also investigated for use in the separation of collagen fragments. Block (triblock) copolymers can self-assemble to form micelle structures in aqueous buffers and can also serve as thermo-responsive gels. Pluronics have many interesting features: they are soluble at low temperature and can gelify with a temperature increase, i.e., the polymer can be easily introduced into the capillaries at a lower temperature and the separation can be carried out at a higher temperature. The 7.5% pluronic F127 (in a pH 2.5, 10 mM Tris, and 75 mM phosphate buffer at 20°C) can be used for the separation of CNBr fragments. The separation is probably a combination of the principles of MEKC and capillary gel electrophoresis, where it resembles the separation achieved by reversed-phase HPLC, but with a different elution order.

Collagenase Fragments

Specific enzymes for the cleavage of collagens are collagenases. There are two major types of collagenase—tissue (interstitial) and bacterial. 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. Because glycine is every third amino acid in collagenous domains, the cleavage of collagen by microbial collagenase resulted in mainly short peptides and a complex mixture (theoretically up to 172 different peptides could arise from a naturally occurring mixture of collagen type I and III).

HPLC

The HPLC separation of peptides arising from the cleavage of collagen by bacterial collagenase is carried out in the “traditional” manner for peptide mapping. The column used is reversed-phase (C18) with normal pore size (10 nm). The gradient used is water–methanol with trifluoroacetic acid as the ion-pairing agent.

Capillary Zone Electrophoresis

The separation of microbial collagenase’ peptides (preferably tripeptides) is not a simple matter for CE; short peptides with proline in the carboxy terminus strongly adhere to the capillary wall. For this reason, both when separating collagenase and CNBr peptides, a very useful method is separation at acidic pH (2.5) using a fused silica capillary, or even the use of a modified capillary (inner surface) by dynamic coating (alkylamines added to the background electrolytes) at acidic pH (one of the best modifiers was 1,7-diaminoheptane).

The capillary electromigration methods can be also successfully used for the determination of the 1/4 and 3/4 fragments of collagens type I and II arising from cleavage by interstitial collagenase. The sensitivity can be enhanced by using a dynamic fluorescence labeling technique (argon ion 488 nm laser) with a running buffer containing 0.05% sodium dodecylsulfate and a noncovalent fluorescent dye for protein, NanoOrange. The collagen (type I or II) and both fragments can be separated and detected within a run time of 20 min by capillary gel electrophoresis (CGE) using a gel buffer (pH 8.8) containing 4% polyacrylamide. The buffer was 50 mM AMPD-CACO (2-amino-2-methyl-1,3-propanediol-cacodylic acid) and a coated capillary was used.

Combination of HPLC and Capillary Electrophoresis

As was mentioned above, the cleavage of tissue consisting of collagen types I and III by bacterial collagenase can result in a rich mixture of peptides (theoretically up to 172 different 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 off-line combination of both methods (CE and HPLC) improves separation—150 peaks were detected.
In the first stage, the peptide mixture was separated by reversed-phase HPLC using gradient elution with a water–acetonitrile system with trifluoroacetic acid as the ion-pairing agent. The collagenous peptides were divided into a few (five or seven) fractions by HPLC. These fractions were further characterized by CE in an uncoated capillary using a phosphate buffer (100 mM, pH 2.5). This two-step peptide mapping with subsequent statistical evaluation (e.g., linear regression analysis) was shown to represent a reliable approach for revealing posttranslational modifications in collagen in vivo.

**Coupling of HPLC and Capillary Zone Electrophoresis with Mass Spectrometry (MS)**

The coupling of HPLC or capillary electrophoresis and mass spectrometry for peptide/protein analysis is a promising technique which will have a significant impact on protein research. Surprisingly, this method is only rarely used for the analysis of collagen. Because collagens are high molecular mass proteins, only the peptidic fragments can be analyzed by mass spectrometry.

The HPLC method uses the procedure described in the section on the separation of collagenase digest—a reversed-phase column (normal pore C18) eluted by a water–acetonitrile gradient with formic acid as the additive. Alternatively, formic acid can be substituted with trifluoroacetic acid (due to its “ion-killing” properties at lower concentrations, e.g., 3 mM). Separation and resolution is slightly better with trifluoroacetic acid as the ion-pairing agent.

Capillary electrophoresis 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 mass spectrometer used was a quadrupole or trap (MS/MS) type. The sheath liquid used was 5 mM ammonium acetate/isopropanol 1:1 (v/v) at a flow-rate of 3 μL/min. This method enables the identification of typical collagenous tripeptides in the collagenase digest, as well as CNBr/trypsin and proteinase K digests.

**CONCLUSION**

Collagen is a broad group of the most abundant proteins in the human body that encompasses at least 28 known types in vertebrates. These proteins are poorly soluble (if at all) and, therefore, they are often split into shorter peptides by enzymatic or nonenzymatic methods before analysis. Collagenous peptides can be successfully separated by high-performance analytical methods—HPLC and CE. The HPLC methods use, most frequently, reversed-phase columns with ion-pairing. Various types of capillary electromigration methods can be used for the separation of collagens and their fragments. The main problem with capillary electrophoretic methods is adhesion of collagenous peptides to the surface of a fused-silica capillary. It can be eliminated by the use of acidic background electrolytes and/or use of surfactants in the separation medium. The high performance method for analysis is combination of HPLC and CE. Nowadays, the high-performance approach is combination of HPLC and CE with MS.

Because collagens are highly important proteins, we can expect that studies of these proteins will be intensified in the future. For these researches we need, and we have, a broad spectrum of analytical methods. However, we can assume that new high-performance methods will be continuously developed.

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**FURTHER READING**


