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Capillary zone electrophoresis of collagen type I CNBr peptides in acid buffers

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Abstract

Collagen type-I CNBr peptides were separated under acidic conditions by capillary electrophoresis. Separation conditions were: 100 m*M* phosphate buffer pH 2.5, 50 cm×50 μm capillary (placed in a cartridge), 8 kV, running time 30–45 min, detection by UV at 200 nm. The peptides were separated strictly by their molecular mass and the overall pattern was well comparable to RP-HPLC separations of these analytes. It is proposed that the separation mechanism may involve hydrophobic sorptions to the capillary wall.

Keywords: Peptides; Collagen; Cyanogen bromide

1. Introduction

Separation of the CNBr peptides represents one of the most widely used methods for collagen analysis both under physiological and pathological conditions. The rationale is based on the fact that the small number of methionine residues leads to a rather limited amount of cleavage products (CNBr peptides). The profile of CNBr peptides is typical at least for the main collagen types and thus provides the appropriate way to estimate the amount, as well as the type of collagen in a particular tissue [1]. So far, there are several methods in use for CNBr peptide analysis, with classical CM cellulose [2] and phosphocellulose [3] chromatography being those with the first priority to be mentioned. The disadvantage of the 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. [4] and van der Rest et al. [5] introduced reversed-phase chromatographic procedures, which exhibited much higher selectivities and shorter analysis time. The most widely used method for CNBr peptides analysis today is, however, gel electrophoretic separation originally introduced by Scott and Veis [6]. This latter procedure is time-consuming and cannot be run in an automated mode and quantitation of the separated peaks is difficult because of the necessity to use staining procedures to reveal the individual peptide zones in the gel. Also, the smaller peptides ($M_r <$ 20 000) are poorly resolved unless a gradient of acrylamide is used [7]. Numerous modifications of gel electrophoresis, as well as other separation

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methods for collagens and their fragments are available today (for review, see Ref. [8]).

While in ion-exchange separations the separation mechanism is based on the charge of individual peptides, in reversed-phase chromatography the separation mechanism is based on the hydrophobicity of individual peptides. Remarkably, this separation follows the molecular mass distribution, the larger peptides being eluted later from the reversed-phase columns than the smaller ones. It is argued that at low pH (2.2) free amino groups of the individual species of both collagen α-chains and collagen fragments are extensively protonated by heptafluorobutyric acid (HFBA) as the ion-pairing reagent, which proved to be the most efficient one and, consequently, all species have a zero net charge. At this low pH silanol groups are protonated; therefore no (or at least negligible) negative charges are expected on the sorbent surface. Collagen exhibits a high internal homology of sequence, in which the tripeptide structural unit Gly-Pro-X (where X may be any amino acid, Gly being the predominatly found species) prevails. This means that any fragment of the parent α-collagen polypeptide will contain roughly the same number of hydrophobic residues. Taken into account that interaction of these hydrophobic moieties with an nonpolar stationary phase is the stronger the greater the number of constituting amino acids, it is obvious that the lower molecular mass fragments elute at low t_r and those with high molecular mass at higher t_r values. This fact can be explained by an increase of the interactive surface of the peptide with increasing chain length yielding a higher degree of overall (apparent) hydrophobicity. This implies that the total hydrophobicity of any collagen fragment will be directly related to its molecular mass. Thus if the governing mechanism of separation is based upon hydrophobicity, one would expect the separation of individual fragments to correlate with their molecular mass. This consideration was first reported by Smolenski et al. [4] in an attempt to explain their results with CNBr peptide separation by reversed-phase chromatography with heptafluorobutyric or trifluoroacetic acids as counter ions.

A perspective method in separating collagens and their CNBr fragments is capillary electrophoresis. To our knowledge this method was used with alkaline buffers at extremely low concentration [9]. This approach was used for the following reasons: to speed up the long-lasting separation and to avoid sticking of the collagen fragments to the capillary wall, bearing in mind that at even moderate salt concentrations collagens and their peptides can relatively easily be salted out. The advantage of the capillary zone electrophoresis lies in its capability to quantitate individual fragments (based on area percentages), in the applicability of automation and in a high resolution power (selectivity). Conversely, low buffer concentrations made the method anything but robust and not easy to transfer from one capillary to another, which, as it appears, depends on the changes in surface chemistry.

Here we report a procedure for separating collagen type-I CNBr peptides by capillary electrophoresis in extremely acid media (pH 2.5) which in our hands was reliable, short and sufficiently selective.

2. Experimental

2.1. Capillary zone electrophoresis

A Bio-Rad (Richmond, CA, USA) capillary zone electrophoresis apparatus HPE 100 equipped with a Microsampler 100 cartridge, 50 cm×50 μm uncoated capillary (catalog No. 148-3014) was used throughout this study. For comparison additional experiments were run with a coated capillary (Bio-Rad catalogue No. 148-3011, linear polyacrylamide coated). All separations were run with a 0.1 *M* phosphate buffer purchased from Bio-Rad (catalogue No. 148-5010), pH 2.5. Routinely 8.0 kV were applied to the positive end of the capillary resulting in 19.2–20 μA current. UV absorption at 200 nm was used for detection. Samples of collagen type-I CNBr peptides were dissolved in water to yield a concentration of 400 μg/ml and were applied electrophoretically using 8 kV for 4 or 6 s. For identification CNBr peptides were isolated by reversed-phase chromatography and lyophilized.

2.2. Reversed-phase chromatography

The liquid chromatograph was composed of two Model 600A pumps, a Model 660 gradient programmer and a Model U6K injector, all from Waters (Millipore, Milford, MA, USA). Columns used were

Vydac TP 201 column (250×4.6 mm I.D., 10 μm particle size, 30 nm pore size). Detection was done by absorbance recording at 200 nm. Elution was done with a linear gradient 12.8–44.8% (v/v) acetonitrile in water, both solvents containing 10 m*M* of HFBA. The flow-rate used was 1.0 ml/min.

2.3. Gel electrophoresis

Gel electrophoretic separations were performed by the method of Laemmli [10] on discontinuous slab gels using 4% stacking gel and 12% separating gel. The gels were stained for 1 h with 0.25% Coomassie Brilliant Blue R in methanol–acetic acid–water (40:10:50, v/v). Destaining was performed for 1 h with methanol–acetic acid–water (40:10:50, v/v). Molecular mass standards were obtained from Sigma (St. Louis, MO, USA) (albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, α-lactalbumin and aprotinin).

2.4. CNBr peptide preparation

CNBr peptides were prepared from collagen type I obtained from Sigma. Cleavage by CNBr was done under N_2 in 70% (v/v) formic acid as described by Scott and Veis [6]. Fragmented collagen samples were lyophilized and dissolved in MilliQ water at a concentration of 400 μg/ml.

2.5. Chemicals

All chemicals used were either of the analytical grade or highest available purity; acrylamide, ammonium persulphate, SDS, N,N,N′,N′-tetramethylenediamine (TEMED) and Tris base were obtained from Sigma. Heptafluorobutyric acid was also a product of Sigma and acetonitrile was purchased from Merck (Darmstadt, Germany). All buffers and solutions were prepared with MilliQ water.

3. Results and discussion

A typical separation of CNBr peptides from collagen type I is shown in Fig. 1. The R.S.D. of migration times was less than 2%. Identification of individual peaks was done by running RP-HPLC

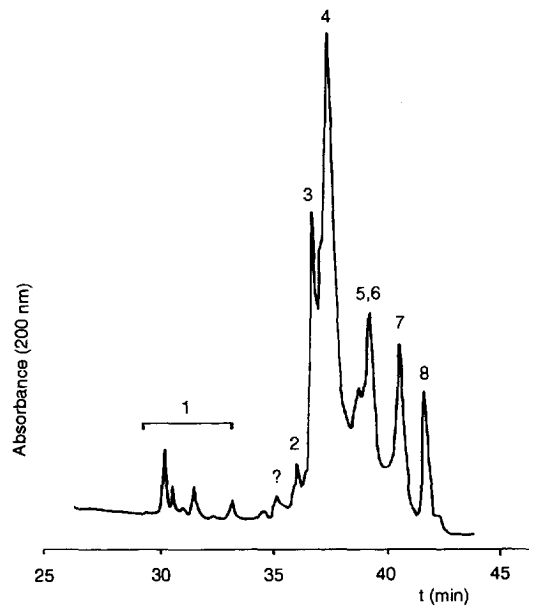

Fig. 1. Elution pattern of the CNBr peptides derived from type-I collagen by capillary zone electrophoresis. Loading conditions: 8 kV, 20 μA, 4 s; run conditions: 8 kV constant voltage, 19.2 μA, +→− polarity, untreated capillary. Identification of peptides: 1=α_1CB_2, α_1CB_4, α_1CB_5, α_2CB_2; 2=α_1CB_3; 3=α_1CB_6; 4=α_1CB_7, α_1CB_8; 5 and 6=incomplete cleavage products; 7=α_2CB_4; 8=$\alpha_2CB_{3,5}$.

isolates on the CE apparatus. All the main peaks, i.e., α_1CB_3, α_1CB_6, $\alpha_1CB_7+\alpha_1CB_8$, α_2CB_4 and α_2CB_{3-5} were identified in this way. It is particularly noticeable that the smaller peptides (α_1CB_4, α_1CB_5, α_1CB_2 and α_2CB_2) are much better separated in the CZE mode as compared to reversed-phase chromatography (see Fig. 2), in which only a set of poorly resolved small peaks can be observed at the beginning of the chromatogram if higher amounts are loaded on the column. On the contrary separation of α_1CB_3 appears better in RP-HPLC than in CZE. The separation of α_1CB_7 and α_1CB_8 is only achieved by CZE; on the other hand these two peptides yielded a single peak in RP-HPLC.

The most remarkable observation here is the similarity of the elution patterns between reversed-phase and capillary electrophoresis. In both cases the individual peptides are separated according to their increasing molecular mass (and increasing hydrophobicity, see Section 1. When these results are compared to slab gel electrophoresis, the sequence of

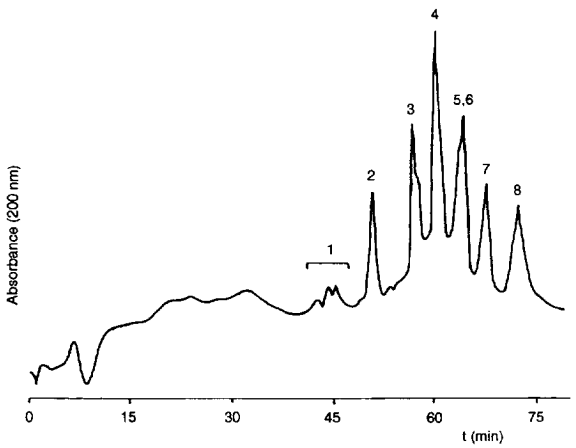

Fig. 2. Reversed-phase HPLC of type-I collagen CNBr peptides (300 μg). Linear gradient from 12.8 to 44.8% (v/v) acetonitrile in water, mobile phase made 10 m*M* with respect to HFBA; flow-rate, 1.0 ml/min. Peak identification as in Fig. 1.

separated peptides again is the same, starting with the low molecular mass peptides at the front of the electropherogram followed by peptides with progressively increasing molecular mass towards the start line (Fig. 3). Because no surfactants were used in the capillary electrophoretic system, no charge equilibration between individual peptides is necessary, which is in contrast to SDS slab gel electrophoresis. It is also unlikely that the separation of CNBr peptides can be attributed to their hydrodynamic resistance towards the electroosmotic flow (in spite of the presence of a polymeric modifier as declared by the producer of the buffer); this opinion is supported by the observation that helix-forming structures (like collagen peptides) tend to move in electrophoretic separations in a snake-like manner with their longer axis in parallel to the flow direction [11]. In addition, it is to be kept in mind that at pH 2.5, at which the separations were performed, most of the silanol groups of the inner surface of the capillary are uncharged. Therefore, the situation in CZE is similar to that in RP-HPLC, i.e., the capillary surface showed to be much more prone to hydrophobic interactions. Consequently, it may be proposed that the separation mechanism in CZE of collagen CNBr peptides is at least in part based on hydrophobic interactions between the peptide moiety and the inner capillary surface. This hypothesis is further supported by the results shown in Fig. 4,

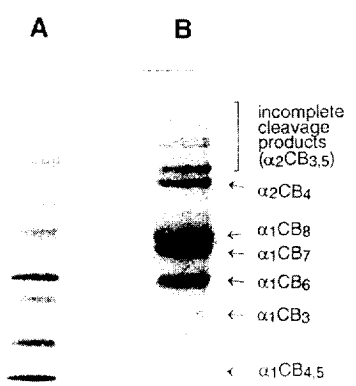

Fig. 3. Comparative profile of collagen type-I CNBr peptides obtained by 12% slab gel electrophoresis as specified in Section 2. (A) molecular mass standards; (B) CNBr peptides.

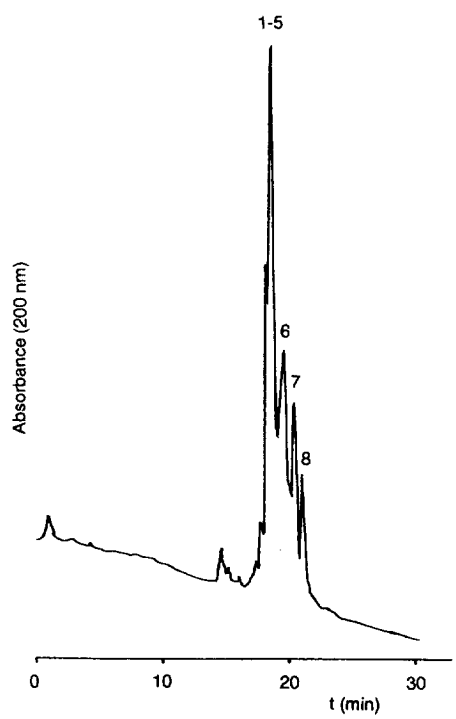

Fig. 4. Comparative profile of collagen type-I CNBr peptides obtained with a surface-treated capillary. Loading and separation conditions as in Fig. 1.

where a surface modified capillary was used for separation under otherwise identical conditions. In spite of the fact the electroosmotic flow is slowed down (7.26 min in the surface-treated capillary as compared to 5.30 min in the surface-untreated column, as demonstrated by benzyl alcohol) the peaks of the peptides appear with a considerably lower retention in the surface treated capillary (the main peak emerges around 18–19 min in the surface-treated capillary, while in the untreated one it appears in the detection window between 36 and 38 min). Also, the selectivity is considerably lower yielding only partial separation of the last three peaks, i.e., peak No. 6 containing incomplete cleavage products, peak No. 7 containing α_2CB_4 and peak No. 8 representing incompletely cleaved peptide $\alpha_2CB_{3,5}$. No separation of peaks attributable to faster migrating components with lower molecular mass values was observed. Ion pairing with heptafluorobutyric acid, which proved to be efficient in reversed-phase separation of collagen CNBr-peptides, had no effect upon separation when this counter-ion was added to the carrier electrolyte. This may be explained by the fact that the peptides as such are hydrophobic enough to ensure a separation mechanism based on hydrophobic interactions and ion pairing with a hydrophobic counter ion is unable to bring about further improvement in selectivity. Conversely, if the carrier electrolyte was made 7.5% with respect to acetonitrile (Fig. 5), slowing down of the whole run was observed as expected. The separation of individual peaks was better particularly in the region of incompletely cleaved peaks (marked No. 5, 6 and ?). Decreasing the voltage resulted in a similar effect (not shown).

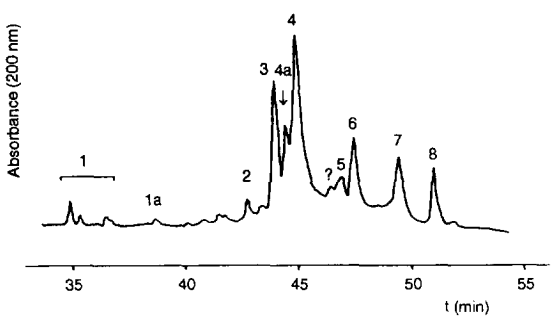

Fig. 5. Comparative profile of collagen type-I CNBr peptides run in a surface untreated capillary but with the addition of 7.5% acetonitrile to the run buffer. Identification of peptides same as in Fig. 1, except for α_1CB_4 (1a) and α_1CB_7 (4a).

It can be concluded that separation of collagen CNBr peptides in capillary electrophoresis using acidic (pH 2.5) buffers and untreated silica capillaries occurs at least in part on the basis of adsorption of the polypeptide chains on the capillary surface, perhaps through hydrophobic bonding. However, ion pairing with a strongly hydrophobic anion (HFBA) remained without any effect. Besides the above consideration this may be explained also on the basis of relatively low amounts of lysine and arginine residues in the collagen molecule. The main contribution to the strong solute-matrix interactions can be ascribed to the peptide's inherent hydrophobicity and not or only to a small degree to the influence of the counteracting ion. Consequently, the peptides are eluted according to their molecular mass, the smaller being first, the large one last on the electrophoregram. So in this case the unfavourable 'sticking' of proteins to the capillary surface can in this case be advantageously exploited for successive desorption by means of endoosmotic flow of the carrier electrolyte and, finally, to the separation of individual collagen derived peptide species. For comparison, polyacrylamide gel electrophoresis of the same sample as separated by CZE in Fig. 1 is shown in Fig. 3.

The role of the pH, as well as concentration of the background electrolyte (and of the ionization of the inner capillary wall) can be further elucidated by taking into consideration previous results obtained at pH 9.5 using 2.5 m*M* sodium tetraborate. It was demonstrated [9] that at alkaline pH and low concentration of the carrier electrolyte (2.5 m*M* borate buffer) CNBr collagen peptides offer strictly linear relationship in the Offord plot [12] namely the relative migration time $t_{m,rel} = -3.25\ M^{2/3}/Z$ (retention related to horse skeletal muscle myoglobin). Therefore it appears reasonable to conclude that under conditions favouring the dissociation of the silanol groups on the inner surface of the capillary the predominating separation mechanism is based on the charge of separated peptides, while at low pH values and higher buffer concentrations sorption (hydrophobic interactions) with the capillary wall predominate.

Acknowledgments

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