Separation of collagen cyanogen bromide-derived peptides by reversed-phase high-performance liquid chromatography

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A new technique for separation of the CNBr cleavage products of collagen is described. It involves the use of a 30nm pore reversed-phase high-performance liquid chromatography column eluted with a linear gradient of acetonitrile/water containing 0.01 M-heptafluorobutyric acid. The separation of type I, type II and type III CNBr peptides is described. Resolution is particularly good for the low-molecular-weight peptides. The method is fast, quantitative and sensitive, and the complete volatility of the eluent facilitates the recovery of the separated peptides.

Cleavage by CNBr is one of the most widely used methods for collagen analysis, especially in pathological specimens. The small number of methionine residues in the collagen molecule results in a small number of cleavage products (CNBr peptides) that vary widely in molecular weight. Their distribution is unique for each collagen type, and this provides a useful means for collagen type identification and structural analysis (Fietzek & Kühn, 1976). Two main methods of separation have been used so far, phospho- or CM-cellulose chromatography (Bornstein & Piez, 1966; Butler *et al.*, 1967) and gel electrophoresis for microanalytical studies (Scott & Veis, 1976). The latter method is not quantitative, because detection is based on the staining intensities of the various peptides and because the smaller sized peptides (mol.wt. less than 20000) are poorly retained by the gel unless an acrylamide gradient is used (Cole & Bean, 1979). Recovery of the separated peptides is not easy with this method. CM-cellulose on the other hand is a tedious method for multiple sample analysis and has questionable recovery. Detection, usually done at 230nm, is not very sensitive, thus requiring relatively large samples.

We report here a new method for separation of the collagen CNBr peptides by reversed-phase h.p.l.c. It combines quantitative detection, at a sensitivity that could previously only have been

obtained by gel electrophoresis, with an easy and complete recovery of the separated peptides.

Materials and methods

Reagents

Acetonitrile (h.p.l.c. grade) was obtained from Fisher Scientific (Montréal, Québec, Canada). Deionized, glass distilled water was purified by passage, under gravity, through a bed of octadecylsilyl-silica made from the content of a C-18 Sep-Pak (Waters Associates, Milford, MA, U.S.A.) (Bennett *et al.*, 1980). Heptafluorobutyric acid (Sequanal grade) was from Pierce Chemical Co. (Rockford, IL, U.S.A.). Electrophoresis reagents and equipment were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Chromatographic equipment

The liquid chromatograph was composed of two model 6000 A pumps, a model 660 gradient programmer and a model U6K injector, all from Waters Associates. Column eluates were monitored for u.v. absorbance at 210nm with a model LC75 spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.). The columns were CN μ Bondapak and C-18 μ Bondapak (3.9mm $\times$ 300mm) from Waters Associates and a C-18 Vydac TP 201 (4.6mm $\times$ 250mm) column from The Separations Group (Hesperia, CA, U.S.A.).

Preparation of the CNBr peptides

Types I and III collagen were prepared from human foetal dermis and type II collagen from

Abbreviation used: h.p.l.c., high-performance liquid chromatography.

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human cartilage as previously described (van der Rest *et al.*, 1977). Cleavage by CNBr was done under N_2 in 70% (v/v) formic acid, as described by Scott & Veis (1976). The type III peptides, kindly given by Dr. E. Golds, had been dialysed against 0.1 M-acetic acid prior to freeze-drying.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis

Electrophoretic separation of the CNBr peptides was performed by the method of Laemmli (1970) on slab gels containing a linear gradient of acrylamide (7–15%, w/v) as suggested by Cole & Bean (1979). The gels were stained for 1 h with 0.25% Coomassie Brilliant Blue R in methanol/acetic acid/water (45:9.2:45.8, by vol.). Destaining was performed in a diffusion destainer with methanol/acetic acid/water (3:2:35, by vol.).

Results and discussion

The technique was established with type I collagen CNBr peptides. Previously, excellent peptide hormone separation had been obtained by using reversed-phase h.p.l.c. columns eluted with acetonitrile/water containing ionic modifiers (Bennett *et al.*, 1980). Therefore we limited ourselves to this system and tried several columns and counter-ions. Poor resolution and early elution of the CNBr peptides were observed when C-18 or CN μ Bondapak columns were tried with either trifluoroacetic acid (0.013 M) or phosphoric acid (0.017 M) as counter-ion. We attributed this behaviour to two factors specific to the collagen molecule: its unusual amino acid composition, with few hydrophobic and charged amino acid residues, and the helical conformation that even CNBr peptides tend to take in solution (Piez & Sherman, 1970a,b). An attempt to modify the fragment conformation by heating the column up to 55°C had no effect on the separation, and neither did the addition of 0.1 M-sodium sulphate. We thus modified the chromatography conditions that were likely to improve the resolution of the CNBr peptides: the nature of the counter-ion and the pore size of the chromatographic support. We chose the larger homologue of trifluoroacetic acid, heptafluorobutyric acid, as counter-ion because it would, through ion-pairing with basic groups, give more hydrophobicity to the peptide (Bennett *et al.*, 1980). We also selected the commercially available C-18 Vydac TP 201 column, since it has a 30 nm pore size instead of 10 nm for the C-18 μ Bondapak column. The use of the larger pores had a favourable effect on resolution, and the more hydrophobic counter-ion markedly increased the retention time and resolution of the CNBr peptides. The optimal conditions were 0.01 M-heptafluorobutyric acid, a 1 h linear gradient

of 12.8%–44.8% (v/v) acetonitrile in water and a flow rate of 1 ml/min. A very slow flow rate might be expected to improve resolution by optimizing the equilibration of peptides with the stationary phase. A 0.25 ml/min flow rate over 2 h did not improve our separation, while a 2 ml/min, 30 min gradient was slightly detrimental.

The separation of the type I collagen CNBr peptides is shown in Fig. 1. The variations noticeable in the baseline during the first 10 min of the chromatography are due to impurities in the heptafluorobutyric acid. Increasing the acetonitrile concentration of the mobile phase up to 80% (v/v) for 1 h did not result in the elution of any other peptide. To identify the eluted peptides, fractions (1.5 ml) were collected in polypropylene tubes between 30 and 66 min of elution, since preliminary experiments had shown that no material detectable on gel electrophoresis would be eluted in the first 30 min. The fractions were dried *in vacuo*, redissolved in 50 μ l of the sample buffer and electrophoresed as described. The protein bands were tentatively identified by comparison with published data (Scott & Veis, 1976). Unlike gel electrophoresis, there is no direct correlation between the molecular weight and the elution time of a peptide, although the retention time tends to increase with the molecular weight. The low-molecular weight peptides were well resolved by our system. Peptides $\alpha 1(I)CB 8$ and $CB 7$ were not resolved, and the

Fig. 1. Reversed-phase h.p.l.c. of type I collagen CNBr peptides (300 μ g) as described in the text

Tentative identification of the peptides with their molecular weights (Scott & Veis, 1976); (a) $\alpha 1(I)CB 2$ (mol.wt. 3300), $\alpha 1(I)CB 4$ (mol.wt. 4400), $\alpha 1(I)CB 5$ (mol.wt. 3500) and $\alpha 2CB 2$ (mol.wt. 2700); (b) $\alpha 1(I)CB 3$ (mol.wt. 13 500); (c) double band on gel electrophoresis [$\alpha 1(I)CB 6$] (mol.wt. 17 400; 20 200 with the non-helical region); (d) $\alpha 1(I)CB 7$ (mol.wt. 24 000) and $\alpha 1(I)CB 8$ (mol.wt. 25 000); (e) and (f) incomplete cleavage products of the $\alpha 1(I)$ chain; (g) $\alpha 2CB 4$ (mol.wt. 29 000); (h) $\alpha 2CB 3,5$ (mol.wt. 60 800).

Fig. 2. Reversed-phase h.p.l.c. of type II collagen CNBr peptides (250 μ g)

Tentative identification of the peptides with their molecular weights (calculated from the data of Eyre & Muir, 1975): (a) two bands corresponding to low-molecular weight peptides on gel electrophoresis; $\alpha 1(\text{II})\text{CB} 6$ (mol.wt. 3300) and incomplete cleavage products involving $\alpha 1(\text{II})\text{CB} 4$, $\text{CB} 2$, $\text{CB} 3$ and $\text{CB} 6$; (b) $\alpha 1(\text{II})\text{CB} 8$ (mol.wt. 13800); (c) four bands on gel electrophoresis which may include $\alpha 1(\text{II})\text{CB} 9,7$ (mol.wt. 9800), $\alpha 1(\text{II})\text{CB} 9,7\text{X}$ (mol.wt. 11000) and $\alpha 1(\text{II})\text{CB} 12$ (mol.wt. 8000); (d) incomplete cleavage product; (e) $\alpha 1(\text{II})\text{CB} 11$ (mol.wt. 25200) and $\alpha 1(\text{II})\text{CB} 10$ (mol.wt. 33100); (f) incomplete cleavage product, $\alpha 1(\text{II})\text{CB} 11-8$; (g) incomplete cleavage product, $\alpha 1(\text{II})\text{CB} 8-10$.

higher-molecular-weight peptides were only partially separated. No attempts were made at this stage to purify, by rechromatography, the peptides that were partially resolved, although this could easily have been done. It should be noted that no CNBr peptide seemed to bind irreversibly to the column. Amino acid analyses of the starting material and of the eluate collected between 30 and 66 min showed that at least 88% of the specimen loaded onto the column was recovered in those fractions. The actual yield was probably higher since this experiment required several transfers and a 360-fold concentration of the eluate.

We also separated the CNBr peptides from type II and type III collagens with this technique, and analysed the collected fractions by gel electrophoresis (Figs. 2 and 3). Tentative identification of the protein bands was done by comparison with the data of Eyre & Muir (1975) and of Cole & Bean (1979). For type II collagen, several small peptides (mol.wt. less than 12000) were detected by gel electrophoresis in the $\alpha 1(\text{II})\text{CB} 9,7$ region, suggesting the presence of the crosslinked peptide $\alpha 1(\text{II})\text{CB} 9,7\text{X}$, of $\alpha 1(\text{II})\text{CB} 12$ and of partial cleavage products of the small peptides. Dissolving unreduced type III collagen was found to be difficult.

Fig. 3. Reversed-phase h.p.l.c. of type III collagen CNBr peptides (250 μ g)

Tentative identification of the peptides with their molecular weights (calculated from the data of Fietzek *et al.*, 1977): (a) double band on gel electrophoresis; $\alpha 1(\text{III})\text{CB} 6$ (mol.wt. 8700) and $\alpha 1(\text{III})\text{CB} 3$ (mol.wt. 8600); (b) $\alpha 1(\text{III})\text{CB} 4$ (mol.wt. 13600); (c) $\alpha 1(\text{III})\text{CB} 8$ (mol.wt. 11500); (d) $\alpha 1(\text{III})\text{CB} 5$ (mol.wt. 21900); (e) incomplete cleavage product; (f) $[\alpha 1(\text{III})\text{CB} 9]_3$ (mol.wt. 62300); (g) incomplete cleavage product.

The use of a 0.13 M-trifluoroacetic acid solution was found to overcome the problem, but this caused the appearance of a solvent-front peak.

The technique described here presents several unique advantages that are potentially very useful for the study of collagen structure. The chromatography is fast (60 min) and re-equilibration of the column takes 15 min. The recovery of peptides loaded on the column is complete. Since the solvent is volatile, the separated fractions can be dried rapidly *in vacuo* and are immediately ready for further study. This is particularly useful for the low-molecular-weight peptides that are easily lost in desalting procedures. Detection at 210 nm is based on the absorbance of the peptide bond and is thus quantitative and highly sensitive; 50 μ g of total CNBr peptides are easily detected. Long term reproducibility is remarkable, since similar separations done several months apart gave identical chromatograms. Preliminary results (M. van der Rest & H. P. J. Bennett, unpublished work) indicate that the technique can be successfully applied to whole-tissue CNBr digests.

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