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SEPARATION OF CYANOGEN BROMIDE PEPTIDES OF COLLAGEN BY MEANS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Cyanogen bromide peptides of bovine collagen Types I, II and III were analyzed using high-performance liquid chromatography (HPLC). Elution patterns of each collagen type were unique and reproducible.

Elution patterns of the CNBr peptides of the $\alpha 1$ and $\alpha 2$ chains of Type I collagen were also unique and together accounted for the major components of Type I collagen.

Analysis of the eluted peptides from HPLC of each collagen type by sodium dodecyl sulphate–polyacrylamide gel electrophoresis showed specific patterns for each collagen. Thus, unique and reproducible HPLC chromatograms were obtained, providing a new analytical method that is simple, sensitive and rapid.

INTRODUCTION

The different types of collagen have been characterized according to elution patterns obtained from ion-exchange chromatography and from electrophoresis. Each type of collagen gives a unique profile when analyzed by these techniques.

Two specific methods for separating collagen CNBr peptides are CM-cellulose[1] and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)[2], each with its inherent attributes. CM-Cellulose provides high resolution but requires a fairly large amount of sample (20–50 mg) and an elution time of 7–8 h[1]. SDS-PAGE is very sensitive, requiring only microgram amounts of sample but takes about 2 days for the electrophoretic, staining and destaining procedures.

High-performance liquid chromatography (HPLC) potentially combines the best features of both methods. It requires only microgram amounts of sample, the results can be obtained in a short time, high sensitivity is inherent and it is relatively simple to employ.

MATERIALS AND METHODS

Collagen isolation

Calf skin and calf tibiae of 2–4 month old animals were obtained from Copaco

(Bloomfield, Conn., U.S.A.). The skin collagens were extracted, purified and separated into the Type I and Type III forms by previously described methods[3]. The epiphyseal cartilage was removed from tibiae by shaving with a Stanley Surform and the cartilage chips were extracted to remove proteoglycans followed by pepsin solubilization and purification of the Type II collagen[4].

The isolated collagens were characterized by amino acid analysis, by SDS-PAGE[5] and by comparison of their CNBr derived peptides by SDS-PAGE[6].

CNBr cleavage

Each collagen was dissolved at room temperature in 70% formic acid at a concentration of 1 mg/ml, under a blanket of nitrogen. A 100-fold (w/w) excess of CNBr to collagen was added, followed by continuous rotary shaking at 30° for 3 h. The reaction mixture was diluted 10-fold (v/v) with distilled water, was aspirated to remove excess CNBr and was lyophilized twice.

HPLC

A Micromeretics 1000 (MIC, Norcross, Ga., U.S.A.) instrument was used with a variable-wavelength UV detector and a chart recorder. Optical density was monitored at 215 nm with full scale absorbance set at 0.2.

A reversed-phase column (250 × 4.6 mm) with LiChrosorb C_{18} (10 μm) packing (Altex) was obtained from Rainin Instruments (Boston, Mass., U.S.A.). It was equilibrated with 10 m*M* potassium phosphate buffer, pH 8.5–acetonitrile (9:1, v/v). All buffers and sample solutions were passed through Millipore filters (0.45 μm) to remove any contaminants.

The samples were dissolved in the above buffer at concentrations of 6–10 mg/ml. A 60–100-μl volume of sample was injected and a linear gradient from 10 to 30% acetonitrile was run, at a flow-rate of either 1.5 ml/min or 0.75 ml/min.

Several variables including column temperature, buffer pH, flow-rate, length of run and gradient curvature (linear or convex) were varied before the final conditions were obtained. The final conditions included: 10–30% acetonitrile linear gradient; absorbance, 0.2 a.u.f.s.; wavelength, 215 nm; chart speed, 40 cm/h; flow-rate, 0.75 ml/min for 2 h, with pressure varying as the gradient developed.

In some experiments 1-ml fractions were collected, their absorbance was determined and the appropriate fractions were pooled. Pooled fractions were quantitatively transferred to Spectrapor 2000 dialysis tubing and were exhaustively dialyzed against 0.1 *M* acetic acid. After dialysis the samples were quantitatively transferred to small test-tubes and were lyophilized twice.

SDS-PAGE

A 50-μl volume of electrophoresis sample buffer was added to dissolve the lyophilized fractions which were then placed at 100° for 5 min. The running gel consisted of a linear gradient of acrylamide–N,N'-methylenebisacrylamide from 7.5 to 15%. The dimensions of the entire gel including a 4% stacking gel were 1 mm thick by 12 cm wide by 14 cm long. The electrophoresis was run at a constant 12 mA, with the voltage changing from 50 to 200 V, until the bromophenol blue dye front emerged from the gel. The electrophoresis was then halted. The gel was stained overnight in a 1% (w/v) Coomassie blue solution, was destained using a 10% iso-

propanol–10% acetic acid (v/v) solution until the protein bands were distinct. The final destain contained the above components plus 10% glycerol (v/v) and the gel was subsequently dried using a gel drier.

RESULTS AND DISCUSSION

Fig. 1 shows distinct differences in the elution pattern of the CNBr peptides of each collagen type. Type I collagen gave the most complex pattern followed by Type III and Type II, respectively. The Type I pattern showed a number of major peaks which continued after the gradient reached 30% acetonitrile; therefore, the elution time was extended for an additional 30 min using 30% acetonitrile. Type II collagen showed two major peaks eluting at approximately 25% acetonitrile, and Type III showed eight major peaks eluting between 20 and 30% acetonitrile. For each type of collagen the major peaks appeared in the last third of the chromatogram although each pattern was distinctly different.

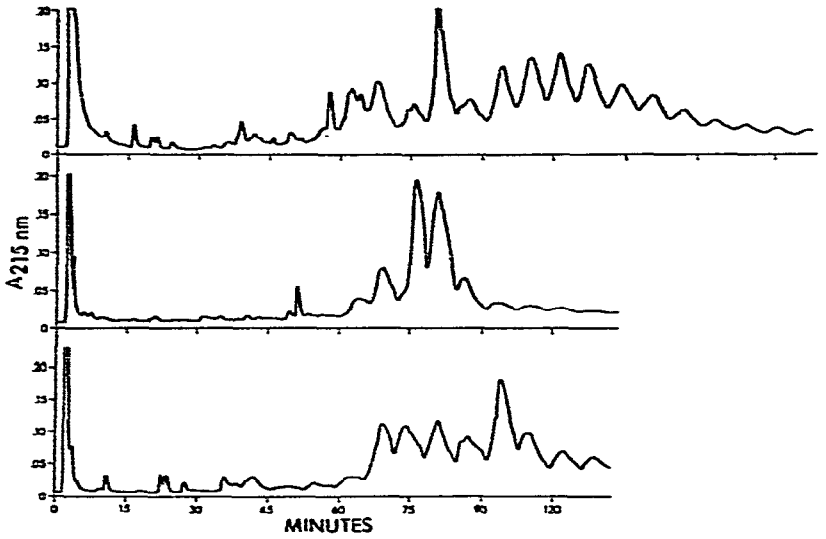

Fig. 1. Chromatogram of the CNBr peptides of different bovine collagens. Upper panel, Type I collagen; center panel, Type II collagen; lower panel, Type III collagen. Flow-rate: 0.75 ml/min, 2 h gradient. All other conditions as described in text.

Fig. 2 shows the comparison of the CNBr peptides of Type I collagen with its component CNBr-cleaved α chains. α1 (I) contributed the major peaks in the early portion of the Type I chromatogram, while α2 contributed to the later portion of the pattern.

The CNBr peptides of Types I, II and III collagen were also eluted under several other conditions. Fig. 3 shows an increase in the flow-rate from 0.75 ml/min to 1.5 ml/min and a decrease in the elution time from 2 h to 1 h (compare to Fig. 1). Both Figs. 1 and 3 show the reproducibility and the uniqueness of each collagen pattern. Fig. 1 shows that better resolution was obtained with a longer gradient time and lower flow-rate.

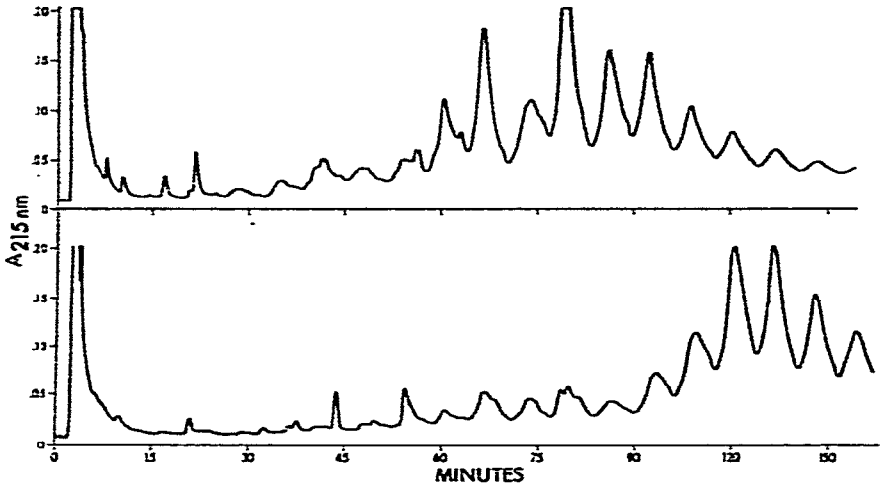

Fig. 2. Chromatogram of the CNBr peptides of the α chains of Type I bovine collagen. Upper panel, α1 (I) chain; lower panel, α2 chain. Other details as in Fig. 1.

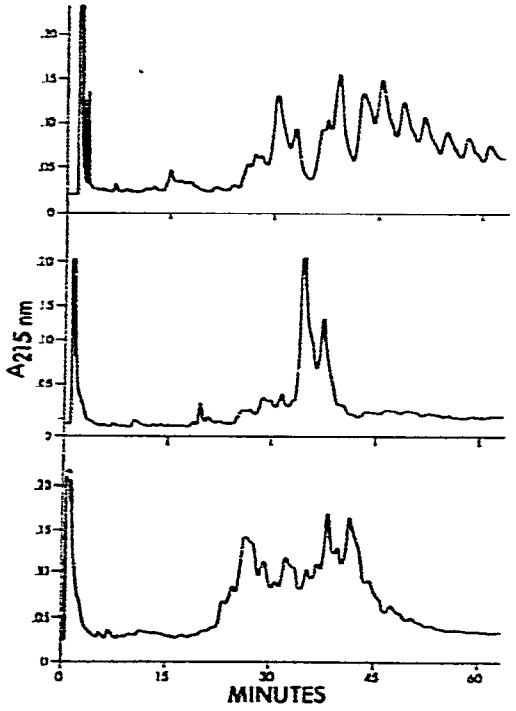

Fig. 3. Chromatogram of the CNBr peptides of different collagens. Upper panel, Type I collagen; center panel, Type II collagen; lower panel, Type III collagen. Flow-rate: 1.5 ml/min, 1 h gradient. All other conditions as described in text.

A blank gradient run was made as a control. A 100-μl volume of buffer solution was injected and all instrument parameters were set as usual. The baseline obtained from this run was straight and steady, showing no peaks other than the initial injection peak.

Parameters such as pH, temperature and gradient curvature were varied. Changing the pH of the buffer from 6.4 to 7.6 or 8.5 showed that the highest pH gave optimum resolution. However, changing the temperature from ambient to 40° gave no further resolution of the patterns. A convex gradient was run from 5 to 30% acetonitrile using CNBr peptides of Type II collagen. The resolution of the small peaks early in the gradient was less clear than with the linear gradient of 10–30% acetonitrile, however, the convex gradient did not change the elution position and resolution of the major peaks.

Since the UV detector has variable absorbance the amount of sample applied to the column can be varied proportionally to the sensitivity. We have obtained a range of peak heights from 0.05 to 0.2 absorbance units without any difference in relative proportions of each component. Samples ranging in amount from 90 μg to 900 μg were used over these absorbance values.

Since the detector is monitoring peptide bond absorbance at 215 nm one would assume peak height to correspond to molecular weight. However, the coelution of several peptides giving rise to a single peak must be considered. SDS-PAGE was done to determine the correlation between the elution pattern and the nature of the emerging peptides. Fig. 4 shows that several major peptides of α1 (I) have eluted. CB 7, CB 3, and CB 4,5 can be seen. Fig. 5 shows several peptides from

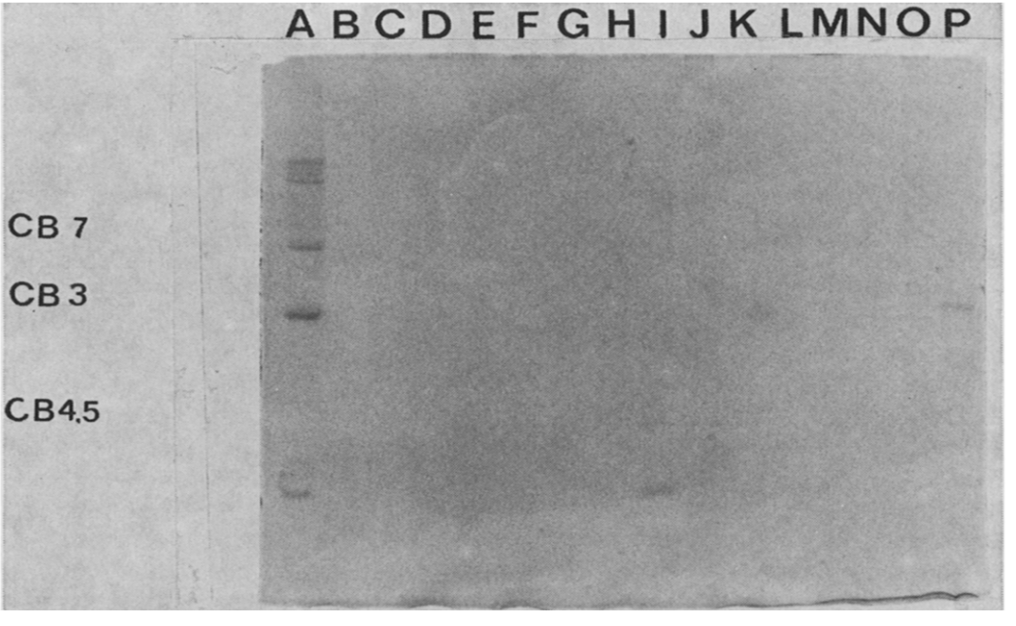

Fig. 4. SDS-PAGE of the eluted CNBr peptides of α1 (I) from HPLC. Slot S contains the pooled eluent from the first 15 min of the chromatogram (Fig. 2, upper panel). Successive peaks were pooled accordingly and are lettered C–P.

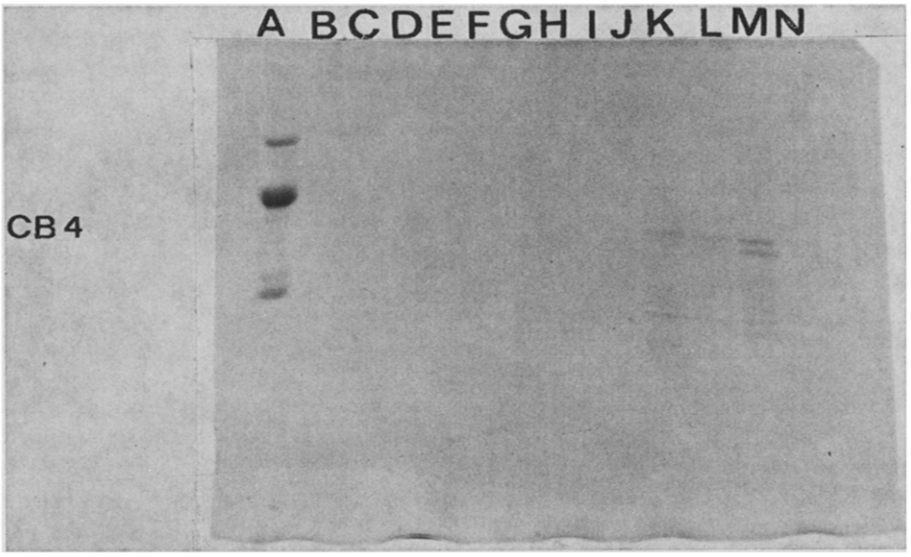

Fig. 5. SDS gel of the eluted CNBr peptides of $\alpha 2$ from HPLC. Slot B contains the pooled eluent from the first 15 min of the chromatogram (Fig. 2, center panel). Successive peaks were pooled accordingly and are lettered C–N.

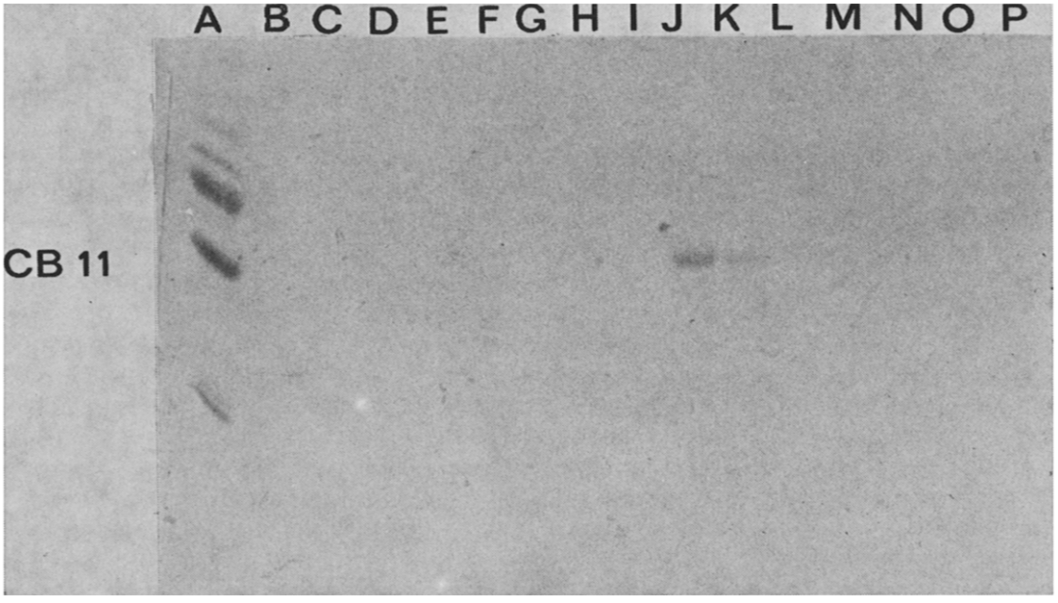

Fig. 6. SDS gel of the eluted CNBr peptides of Type II collagen from HPLC. Slot B contains the pooled eluent from the first 30 min of the chromatogram (Fig. 1, center panel). Successive peaks were pooled accordingly and are lettered C–P.

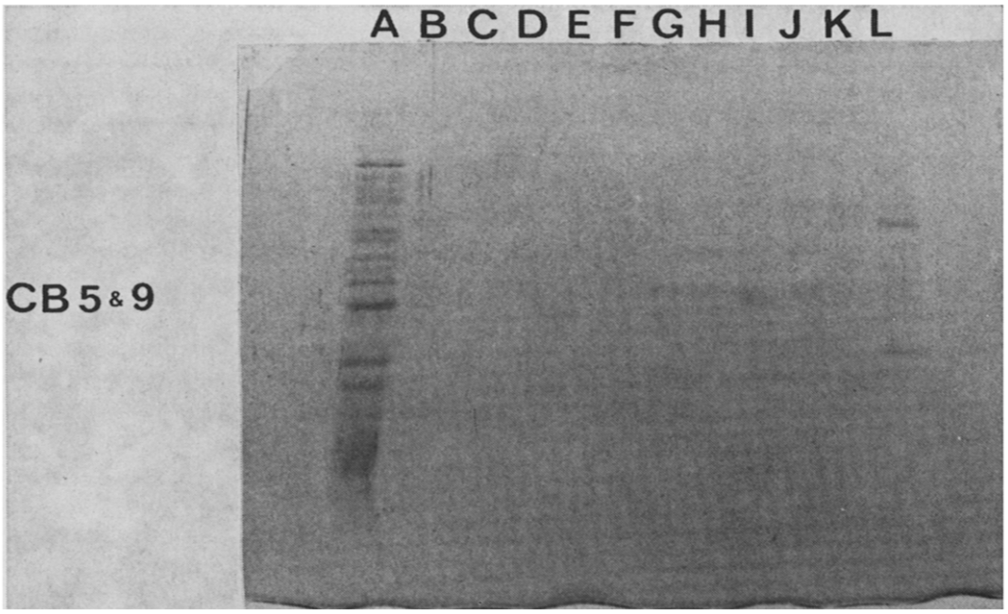

Fig. 7. SDS gel of the eluted CNBr peptides of Type III collagen from HPLC. Slot B contains the pooled eluent from the first 15 min of the chromatogram (Fig. 1, lower panel). Successive peaks were pooled accordingly and are labeled C–L.

the later portion of the α2 chromatogram that are visible on the gel. Among these peptides, CB 4 is the most prominent. Fig. 6 shows the eluted peptides of Type II collagen compared to the initial mixture. CB 11 can be seen and appears in J and K slots corresponding to the two large sequential peaks in the chromatogram. Fig. 7 shows that several peptides of Type III collagen have eluted. CB 5 + 9 can be seen in slots G–L along with many other CNBr peptides.

It is obvious from the gel data that not all peptides have eluted. It is known that low recoveries are common due to irreversible binding to the column through non-specific hydrophobic interactions[7]. Other small peptides of each collagen type may have eluted but the amounts recovered were too small to be detected on the gels. As seen in Fig. 4–7 several sequential fractions contain the same peptide. This is particularly noticeable for Type I collagen which has the most complex elution pattern. The reason for this phenomenon is unknown but the formation of homoserine lactone might be a contributing factor.

The elution times and peak maxima for each collagen type as well as those for the isolated α_1 and α_2 chains of type I collagen are given in Table I. Comparison of the elution profile and derived data of a CNBr digest of an unknown collagen type with the known profiles and with the data in Table I will allow the rapid identification of the collagen being analyzed.

Despite the above limitations the use of HPLC for the separation of collagen CNBr peptides proves to be a new qualitative method for the identification of the different collagen types.

TABLE I

ELUTION TIMES AND RELATIVE PEAK HEIGHTS OF THE CNBr DIGESTS RUN ON HPLC AS SHOWN IN FIGS. 1 AND 2

Peak heights are relative (as a percentage) to the largest peak eluted in each run and are designated as follows: × = peaks greater than 10% but less than 30% of the maximum peak; ×× = peaks greater than 30% but less than 60% and ××× = peaks greater than 60% of the maximum peak.

Peak elution time (min)	*Collagen*				
	Type I	*Type II*	*Type III*	*α_1 Type I*	*α_2 Type I*
10.3	×		×	×	×
16.6	×				
19.7	×				
21.3	×		×	×	
23.7	×		×		
35.5	×		×		
38.7	×				
41.8	×		×	×	
45.0				×	×
49.7	×	×			
55.8				×	×
57.6	××			×	
62.4	××			××	×
63.9	××	×	×	×	
67.9	××	××	××	×××	×
75.0	××	×××	××	××	×
80.5	×××	×××	××	×××	×
87.6	××	××	××	×××	
93.9	×××	×	×××	×××	×
100.3	×××	×	××	××	××
106.6	×××		×	×	××
112.1	×××		×	×	×××
119.2	××			×	×××
126.3	××				××
132.6	×				
139.7	×				
146.1	×				
152.4	×				

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