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SEPARATION OF COLLAGENS BY CAPILLARY ZONE ELECTROPHORESIS

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SUMMARY

Collagen (types I, II, V, IX and XI) constituting polypeptide chains and their polymers and cyanogen bromide-cleaved peptides of collagen type I and type III were investigated by means of capillary zone electrophoresis. Separations were effected in 2.5 mM sodium tetraborate buffer in less than 15 min. A 50 cm × 0.1 mm I.D. fused-silica capillary was used. The separations were run at 18 kV per capillary. The results of the separation were monitored at 220 nm with an on-tube detection system. Using the Offord equation, relative retention times of cyanogen bromide cleavage fragments were plotted against M^{2-3}/Z, were M is the molecular mass of a polypeptide and Z its valency. A linear relationship was observed. Collagen α-chains and their polymers were also satisfactorily resolved.

INTRODUCTION

Collagens represent a family of proteins with a common building scheme but different in molecular size and charge[1]. In addition, some collagen types contain non-collagenous domains which occur either terminally or inside the molecule. Such domains can affect the behaviour of the molecule during chromatographic or electrophoretic separations. Routinely, collagens are separated by polyacrylamide gel electrophoresis, using slab gels according to Laemmli[2]. This system works with sodium dodecylsulphate (SDS) and slightly alkaline buffers. Alternatively, although not frequently used, is the separation of collagens in acid polyacrylamide gels. In this instance the polarity of the run must be reversed, *i.e.*, with a positive pole on the top of the gel[3].

Collagen molecules consist of three polypeptide chains which may differ in their primary structure and, moreover, can be polymerized. This yields multi-component mixtures for the separation for which the application of high-performance separation procedures is desirable. There are two possibilities that can be exploited, *viz.*, high-performance column chromatography[4] or capillary zone electrophoresis. Successful

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separations of collagen polypeptide chains and chain polymers have been obtained by high-performance liquid chromatography with a variety of reversed phases[5,6].

Capillary zone electrophoresis has not previously been applied in collagen separations, but in the light of recent reports dealing with proteins it seems very promising[7–9]. The aim of this study was to establish the conditions under which different types of collagen, collagen chain polymers or collagenous cyanogen bromide-cleaved (CNBr) peptides can be separated by capillary zone electrophoresis.

EXPERIMENTAL

Collagens and collagen CNBr peptides

Collagens type I, II, V, IX and XI were used. Collagen type I was isolated as either the acid-soluble fraction (ASC) or insoluble fraction, solubilized under denaturing conditions (42°C, 30 min). Other collagen types were isolated by pepsinization. Detailed isolation procedures have been published elsewhere[10,11]. The identity of individual collagen types was verified by polyacrylamide gel electrophoresis, amino acid and carbohydrate composition. CNBr cleavage of collagen type I and III α-chains and their purification followed well established procedures[12,13].

Chemicals

Sodium tetraborate of analytical-reagent grade was purchased from Lachema (Brno, Czechoslovakia) and used for the preparation of a 2.5 m*M* buffer (pH 9.2). For the separation of CNBr peptides the pH was increased to 10.5. When necessary, the pH was adjusted with 0.1 *M* sodium hydroxide solution. Before use, the buffer was degassed by heating, cooled and ultrafiltered. Samples of collagen were prepared at a concentration of 1 mg/ml by dissolving lyophilized collagen in the running buffer.

Equipment

Capillary zone electrophoresis was effected in a laboratory-assembled apparatus[14] resembling the set-up published by Jorgenson and De Arman-Lukacs[15]. An untreated fused-silica capillary protected on the external surface with a silicone-rubber layer (Institute of Physics, Slovak Academy of Sciences, Bratislava, Czechoslovakia) was used for separation. The capillary was 50 cm long (to the detector) with I.D. 100 μm (with an additional 10 cm to the cathode). Before analysis the capillary was washed with 1 ml each of methanol, chloroform, methanol, 1 *M* sodium hydroxide solution and 3 *M* hydrochloric acid. Finally, the capillary was washed with 2 ml of the running buffer, attached to the voltage source and left running at 10 kV until the current dropped to 20 μA.

Nearby the cathodic end of the capillary the plastic covering sheet was removed to a length of 1 cm and this part was used for detection.

The light beam of the UV detector was passed through a slit and was generated by a deuterium lamp. Measurements were made at 220 nm.

The capillary was attached to a variable-voltage source. All runs were performed at 18 kV and 25 μA. If the current exceeded 30 μA, the voltage automatically dropped to avoid overheating. The separation time never exceeded 15 min.

Samples of denatured collagen (5 min, 50°C) were loaded from a sample vial electrophoretically. The sampling vial was attached to the anode and had a volume

0.5 ml. At the beginning of the analysis the capillary was inserted in the sampling vial and the sample was introduced at 10 kV for 6 s. Subsequently, the voltage was disconnected and the end of the capillary was inserted into the anode jar.

RESULTS

The results indicate that capillary zone electrophoresis is capable of resolving single collagen chains, chain polymers and CNBr fragments. As shown in Fig. 1A, the electrophoretic profile can be divided into three regions, corresponding to α-chains, their dimers (β-chains) and higher polymers (γ-chains). In the α-chain region a distinct separation of α_1- and α_2-chains of type I collagen is demonstrated. Similarly, in the β-region two distinct peaks of β_{12}- and β_{11}-chains are seen. The identity of individual zones is further verified in Fig. 1B–D: collagen α-chains and their β- and γ-polymers isolated from a polyacrylamide gel run are separated here by capillary zone electrophoresis. The difference seen in the profile of the γ-fraction reflects the increased amount of the sample applied. The polydispersity of this fraction is evidently caused by different proportions of α_1- and α_2-chains constituting chain trimers.

Fig. 2A shows the separation of insoluble collagen solubilized under denaturing conditions. As expected, the proportion of chain polymers in this mixture is considerably increased in comparison with acid-soluble collagen (Fig. 2B). Addition of a submicellar concentration of SDS (0.5 m*M*) to both the sample and the running buffer results in the disappearance of the fine structure of the peaks in the α- and β-regions (Fig. 2C).

Fig. 3 shows the results obtained with some other collagen types. Fig. 3A shows the separation of collagen type II with the molecular formula $[\alpha_1(II)]_3$, yielding single

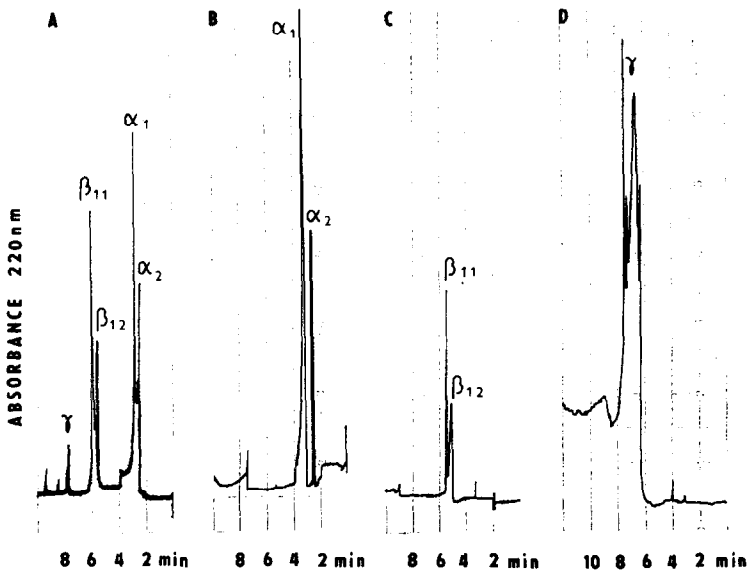

Fig. 1. Separation of acid-soluble collagen (*i.e.*, type I) (A) Denatured molecule; (B–D) individual fractions isolated from polyacrylamide gel electropherograms.

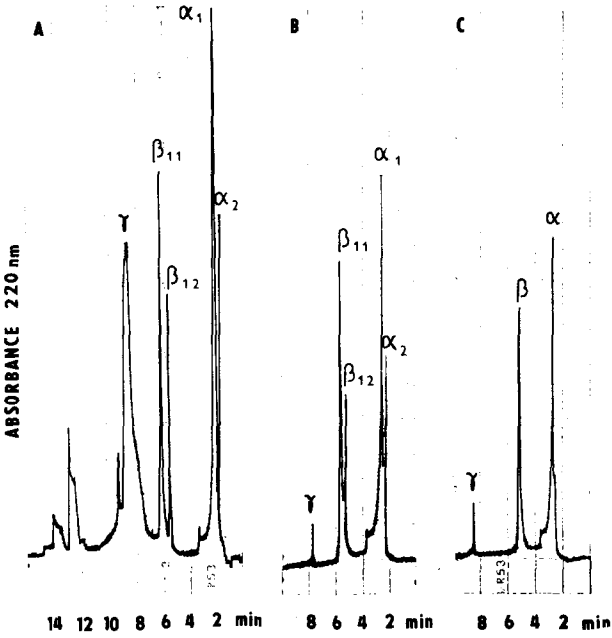

Fig. 2. (A) Separation of insoluble collagen solubilized under denaturing conditions (42°C, 30 min); (B) isolated collagen type I; (C) same sample as in B, run in the presence of 0.5 m*M* SDS.

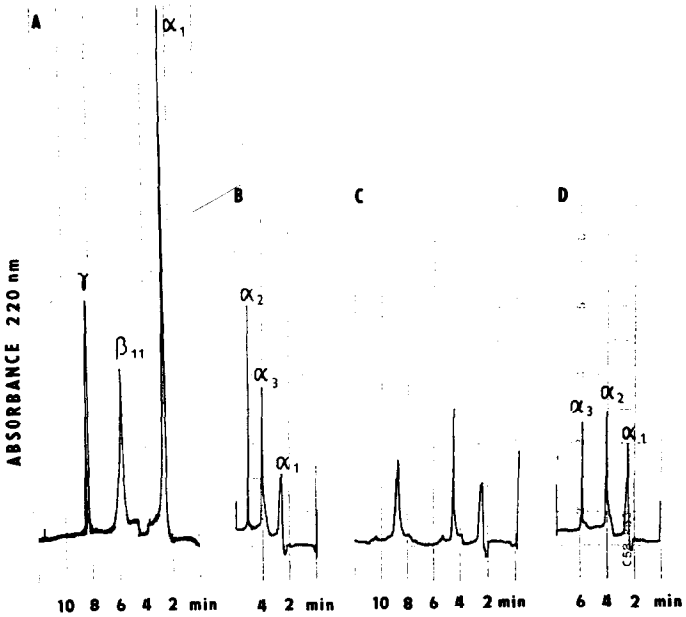

Fig. 3. Separation of collagens (A) type II, (B) type V, (C) type IX and (D) type XI.

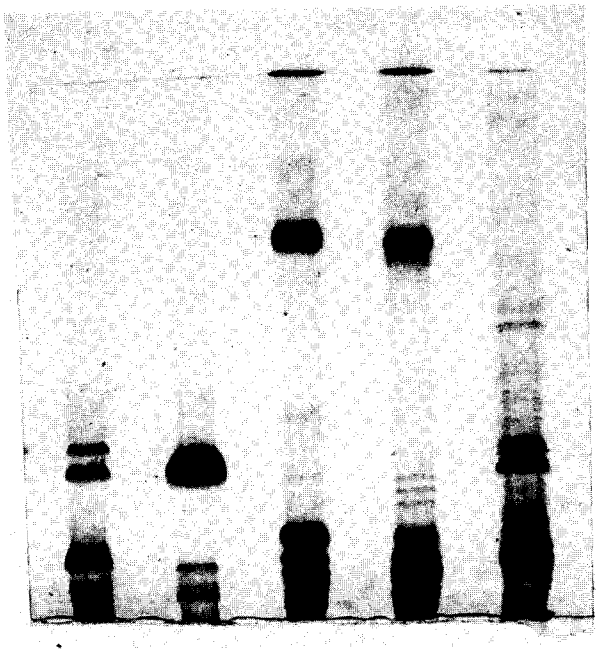

Fig. 4. Polyacrylamide gel electrophoresis of collagen samples used in capillary zone separations. From left to the right: acid-soluble collagen (type I), collagens type II, type IX, type XI and insoluble collagen solubilized under denaturing conditions.

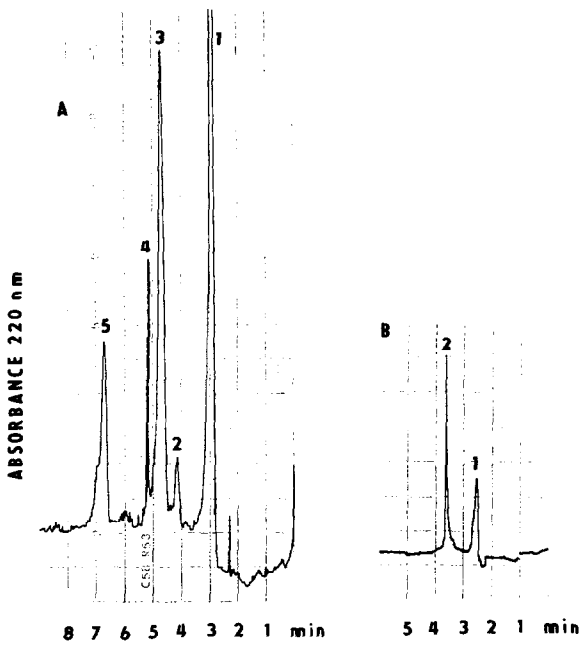

Fig. 5. Separation of CNBr peptides. Peptides arising from (A) α_1(I) and (B) α_1(III) chains of collagen. Peaks: (A) 1 = α_1(I)CB_6; 2 = unknown; 3 = α_1(I)CB_7; 4 = α_1(I)CB_8; (B) 1 = a mixture of α_1(III)CB_4 and α_1(III)CB_5; 2 = α_1(III)CB_9.

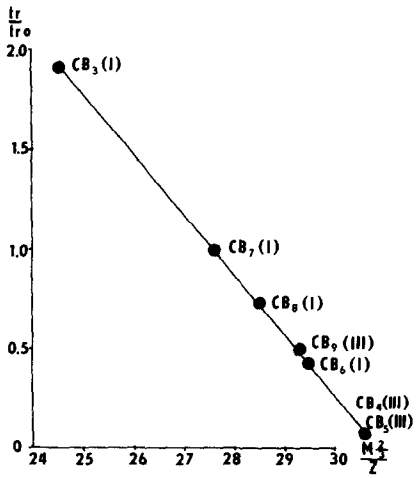

Fig. 6. Relationship between relative retention time (t_r/t_{ro}) and M^{2-3}/Z in Offord's equation for individual CNBr peptides investigated[16].

peaks in the α-, β- and γ-regions). Fractionation of α_1(V), α_2(V) and α_3(V) is shown in Fig. 3B. Owing to the special structure of type IX collagen, fragments different from α-chains arise on pepsinization. The situation is further complicated by the possibility of polymer formation. Therefore, the profile of collagen type V components is different from that of type I or type II collagen (Fig. 3C). The potential of capillary zone electrophoresis was further demonstrated by separating collagen type XI constituents. Three peaks correspond to the three different polypeptide chains of type XI collagen.

The results obtained by capillary zone electrophoresis are in good agreement with the profiles obtained by polyacrylamide gel electrophoresis, as demonstrated in Fig. 4.

TABLE I

NUMERICAL VALUES OF PARAMETERS IN OFFORD'S EQUATION AND PARAMETERS CHARACTERIZING MODEL PEPTIDES

Collagen	*Peptide*	*Retention time (min)*	*Relative retention time (relative to CB7)*	*Molecular mass (M)*	*No. of acidic residues (Z)*	M^{2-3}/Z
Type I	CB_3[a]	8.90	1.91	14 500	23	24.65
	CB_6	3.40	0.43	20 000	25	29.44
	CB_7	4.70	1.00	24 000	30	27.73
	CB_8	4.07	0.73	25 100	30	28.56
Type III	CB_4	2.51	0.05	13 000	18	30.66
	CB_5	2.50	0.05	20 000	24	30.70
	CB_9	3.50	0.48	21 100	26	29.34

[a] When in a complete mixture, peptide α_1(I)CB 3 moves with a retention time of 6.8 min.

Separation of CNBr peptides arising from α_1-chains of collagen types I or III is shown in Fig. 5. Fig. 5A demonstrates the profile of α_1(I)CNBr peptides and the fragments arising from collagen type III are shown in Fig. 5B.

Relative retention times evaluated using Offord's equation[16,17] show a linear dependence on the magnitude M^{2-3}/Z, where M is the relative molecular mass of the peptide and Z its valency (number of negative charges in the molecule) (Fig. 6). The data necessary for calculating the relationship shown in Fig. 5 are summarized in Table I. The calculation of the relative mobility of individual solutes was corrected for endoosmotic flow by deducting the retention of an unretained solute (2.4 min).

DISCUSSION

Capillary zone electrophoresis has proved useful for the separation of collagen polypeptide chains and their polymers. Satisfactory results were obtained with different collagen types. The separations are fast; most of them can be carried out in less than 15 min. The optimum concentration of the samples applied ranges from 0.2 to 2.0 mg/ml and full-scale peak size can be obtained with 10–15 amol of the protein. This opens up new horizons in determining extremely small amounts of collagen.

Although separations were routinely run at 18 kV per 50-cm capillary, satisfactory results were obtained at 10–15 kV, but the separation time was proportionally increased. Overloading of the capillary with sample should be avoided as otherwise poorly reproducible electropherograms are obtained. One of the reasons may be length of the sample applied. The other reason for such irregularities may be a consequence of adherence of protein to the capillary wall at high protein concentrations; clusters of adhering protein seem to be released at a later stage of the separation owing to the endoosmotic flow causing irregular spikes on the electropherogram. If, however, the amount of sample applied is kept within the specified limits, such effects are not observed and the high pH (9.6) of the electrophoresis buffer is capable of preventing adsorption of protein on the capillary wall. The nature of the buffer used is also of considerable importance. Whereas the separation of type I collagen into α-, β- and γ-fractions can be obtained, *e.g.*, with phosphate buffer at nearly the same pH, attempts to separate further α_1- from α_2-chains and β_{11}- from β_{12}-chains under these conditions were unsuccessful. Capillary zone electrophoresis also proved to be useful for the separation of CNBr peptides arising from individual collagen α-chains. The relative retention is linearly dependent on the factor M^{2-3}/Z and fits Offord's equation[16].

The main difference between the results obtained with capillary and polyacrylamide gel electrophoresis is in the application of SDS. Whereas in SDS polyacrylamide gel electrophoresis clear separations of α_1- and α_2-chains are routinely obtained, in capillary zone electrophoresis the addition of 0.5 mM SDS to the running buffer leads to the formation of a single peak containing both α_1- and α_2-chains. This can be explained on the basis of charge difference equilibration of the randomly coiled collagen polypeptide chains in the presence of SDS. Hence it can be concluded that the separation of collagen samples into α-, β- and γ-fractions in capillary electrophoresis reflects primarily the hydrodynamic conditions of the separation. Consequently, it is feasible to propose that the separation of α_1 and α_2 polypeptide chains (and also β_{11}- and β_{12}-chains) is governed by delicate charge differences which, however, can manifest themselves only under specific conditions of optimized separation.

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