Separation of collagen type I chain polymers by electrophoresis in non-cross-linked polyacrylamide-filled capillaries

Z. Deyl[a,*], I. Mikšík[b]

[a]*Department of Analytical Chemistry, Institute of Chemical Technology, Technická 1905, CZ-16228 Prague 6, Czech Republic*
[b]*Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220 Prague 4, Czech Republic*

Abstract

Gel electrophoresis in 4% non-cross-linked polyacrylamide and 35 cm (to the detector) $\times 75$ μm I.D. capillaries was demonstrated to be suitable for separating collagen type I α-chains, chain polymers of the β- and γ-size (dimers and trimers of α-chains) as well as chain polymers larger than γ. Separations were run in 50 mM Tris–glycine buffer pH 8.8 with the sampling port at the anodic end of the capillary. Detection was done routinely by UV at 220 nm. By this method it was possible to demonstrate the differences in α-chain polymer profiles after incubation of the samples with glucose.

1. Introduction

Collagens represent a family of closely related proteins (about nineteen types have been recognized so far), of which collagens type I, II and III represent the most widely distributed species in the animal body (for review see Ref. [1]). Collagen molecules of the most common species, collagen type I, are constituted of three polypeptide chains named α_1 (two chains) and α_2 which are accessible to polymerization through intra- and intermolecular bonds both in vitro and in vivo. Analyzing of these chain polymers is, aside to collagen type identification, the main task in studying the biological functions of these proteins.

The methods generally applied for separation and identification of collagens are: differential salt precipitation [2], CM-cellulose chromatography [2] and CNBr-cleaved peptide analysis [3]. All these methods are applicable only if a large amount of sample is available. However, the sample amount can be considerably lower if gel electrophoresis is used. As a matter of fact sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis as introduced by Hayashi and Nagai [4] represents today the routine method of collagen polypeptide chains analysis. The method is capable of distinguishing not only the major constituting peptide chains (α_1 and α_2) of collagen type I, but also chain polymers (β_{12}, β_{11} and γ). The fact that γ-chain polymers can be distinguished by this method opens the possibility of distinguishing between collagen types I

---

* Corresponding author. Permanent address: Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220 Prague 4, Czech Republic.

and III. Nevertheless, with this method analyzing of higher-chain polymers than γ appears impossible.

With the increasing use of capillary electrophoresis [5] attempts have been made to separate collagen chain polymers by this method [6]. Though basically successful the main problem was that for good separations it was necessary to work at low buffer concentrations (2.5 mM borate, pH 9.2) which, on the other hand, led frequently to sticking of the collagen polypeptides to the capillary wall. Still, however, the advantages of capillary electrophoresis over the flat bed gel arrangement are obvious and are based mainly on the possibility of quantitating the separated proteins which in flat gel electrophoresis may be difficult and not sufficiently reliable. Moreover, in capillary electrophoresis there is a chance to separate higher-chain polymers than γ, if present. This may be of utmost importance when non-enzymatic posttranslational modifications of collagen are studied; glycation and glucose-derived cross-links, the presence of which is assumed in e.g. diabetic or aged tissues, may represent typical examples.

In this study we describe the application of capillary electrophoresis using non-cross-linked polyacrylamide for the separation of collagen type I polypeptide chains and chain polymers. Both naturally occurring and glycation-induced chain polymers were analyzed.

2. Materials and Methods

2.1. Capillary zone electrophoresis

Fused-silica capillaries, untreated, 45 cm long (35 cm to the detector) ×75 μm I.D., purchased from Polymicro Technologies (Tucson, AZ, USA) mounted into an ISCO (Lincoln, NE, USA) capillary electrophoresis apparatus were used throughout this study. All separations were run in 50 mM Tris–glycine buffer pH 8.8. Routinely 10 kV were applied on the capillary; absorbance at 220 nm was recorded (temperature ambient, current less than 50 μA).

Gel-filled capillaries were prepared by the modified procedure described by Yin et al. [7] in the modification of Wu and Regnier [8]. Briefly, a 5-ml volume of 4% acrylamide solution was degassed by aspiration 30 min; then 100 μl ammonium persulphate (APS; 10%), 20 μl N,N,N',N'-tetramethylethylenediamine (TEMED, 10%), and 50 μl SDS (10%) were added. After stirring the solution was fushed into the capillary by pressure application. The column was let standing at room temperature for 1 h and was ready to use.

2.2. Collagen sample preparation

Collagen type I was purchased from Serva (Heidelberg, Germany); a 100 μg/ml solution was prepared in 50 mM sodium phosphate buffer pH 7.2. Then SDS was added to obtain a protein:tenside mass ratio 1:2.5 which ensures complete absorption of SDS to proteins [9]. SDS–protein samples were electrophoretically injected into the gel column by setting the anodic end of the column into the sample vial and applying 10 kV for 10 s.

Glycated collagen samples were prepared by adding 200 μg/ml glucose to a collagen solution containing 100 μg/ml of the protein and prepared in 50 mM sodium phosphate buffer pH 7.2. Incubation was carried out for indicated periods time at 37°C in the presence of sodium azide.

2.3. Chemicals

All other chemicals, i.e. acrylamide, APS, SDS, TEMED, Tris base and glycine were obtained from Bio-Rad (Richmond, CA, USA). All buffers and gel solutions were prepared in Milli-Q water.

2.4. Slab gel electrophoresis

Slab gel electrophoresis was done according to Smolenski et al. [10].

3. Results and Discussion

As shown in Fig. 1, SDS electrophoresis in capillaries containing non-cross-linked poly-
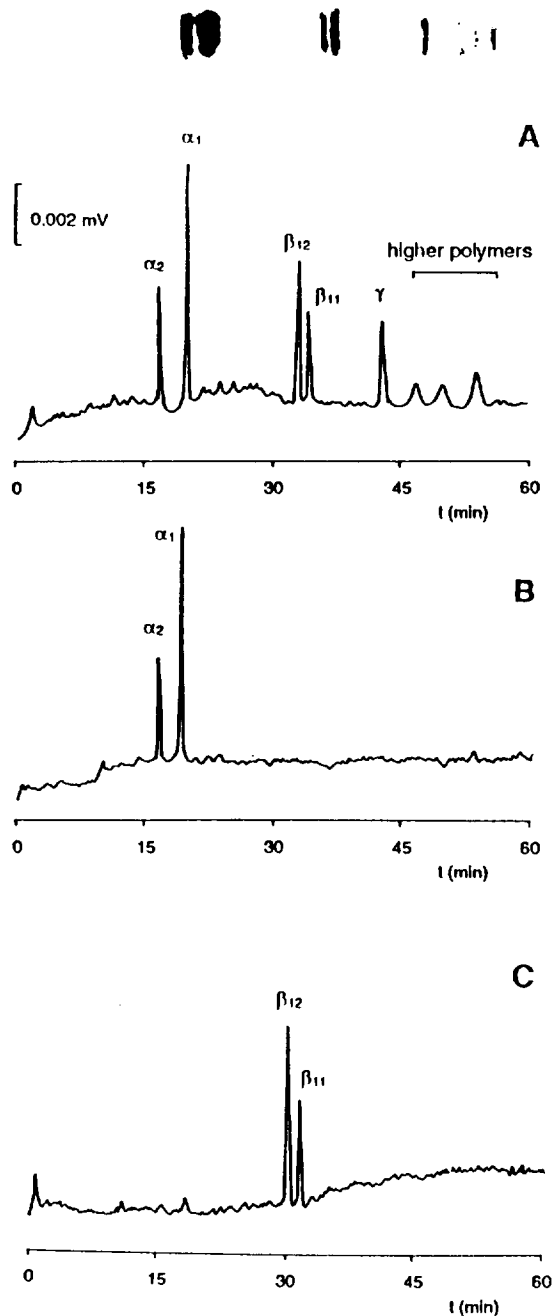
Fig. 1. Capillary gel electrophoresis profiles of collagen α-chains and chain polymers in 4% polyacrylamide; 45 cm long (35 cm to the detector) × 75 μm I.D. capillary; Tris–glycine buffer pH 8.8, 10 kV. Detection by UV absorbance at 220 nm. (A) Complete sample; (B) α-region proteins sampled from a preceding slab gel run; (C) as (B), but β-region excised from the slab gel. The appearance of the gel separation is shown on top of the figure (cathode on the right side of the gel).

acrylamide offers separations of constituting α-polypeptide chains as well as their polymers which are at least comparable with slab gel electrophoresis. Moreover, it offers to quantitate the area percentage of individual peaks, a procedure which is difficult to perform on the slab gel.

Identification of the peaks shown in Fig. 1A was done by running the same sample on a slab gel which was divided into two parts; one part was stained with Coomassie Brilliant Blue, the other was cut into two areas containing the α- and β-chains, briefly homogenized with minimum amount of the run buffer, transferred into a vial and used as sample for the capillary electrophoretic run.

As shown also in Fig. 1, capillary electrophoresis in non-cross-linked gels offers the possibility of separating chain polymers of the γ-chain size (300 000) and higher. This is of considerable importance because no method offering sufficient selectivity is available for analyzing such polymers. Even in diluted polyacrylamide slab gels, these high-molecular-mass fractions either stick to the starting line or move as a smear between γ-fraction and the start. Other methods, like Sepharose gel permeation chromatography don't offer sufficient selectivity (for a review see Ref. [9]).

With collagen type I sample incubated with glucose, the results seen in Fig. 2 can be obtained. There are two features in which such profiles differ from untreated collagen electropherograms, namely in splitting the α-peaks into two each, and in the increased peaks of γ-chain polymers and higher. Both these effects can be ascribed to the interaction of free lysine aminogroups with glucose. It has been shown by Reiser et al. [11,12] that such interactions are non-specific affecting several lysine residue in the collagen molecule and upon prolonged incubation lead to progressive insolubilization of the collagenous proteins.

Based on these observations it is feasible to assume that splitting of the two α-chain peaks into four upon incubation may reflect monotopical binding of glucose to several sites of collagen α-chains which should result in a decrease of positive charges of these polypeptide chains,
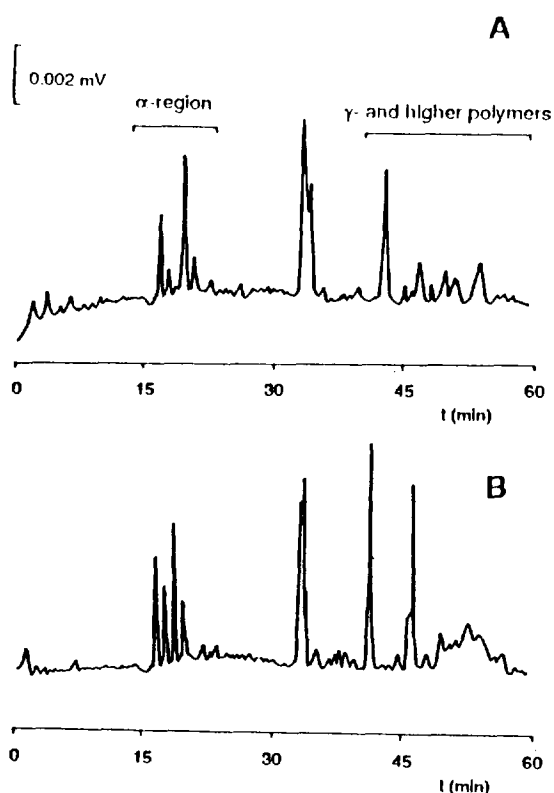
Fig. 2. Capillary gel electrophoresis profiles of collagen α-chains and chain polymers in 4% polyacrylamide; after incubation for 4 days (A) and 7 days (B) at 30°C. Other conditions as in Fig. 1. Note the splitting of peaks in the α-region and the increase of γ- and higher chain polymers. Unincubated profile (control) is shown in Fig. 1A.

changes in hydrophobic properties of the molecule and possibly some conformational changes as well; the higher proportion of γ-chain polymers (and higher) could be explained on the basis of the glucose-based polymerizing reactions which eventually lead to the decreased solubility of incubated collagen samples (for review see Ref. [11]).

To explain splitting of the α-polypeptide peaks upon incubation is difficult at the moment. It is well documented that collagen polypeptide chains, apparently due to their unique primary structure, behave anomalously in polyacrylamide gel electrophoresis (for exhaustive treatment see Ref. [13]). Glycation of a collagen α-chain not only decreases the number of positive charges,

but introduces a relatively large sugar molecule which may further change the number of ionizable side chain groups (OH functionalities in the sugar moiety) and/or change the hydrophobic properties of the parent α-chain. It is also not yet clear whether glycated or non-glycated α-chains bind the same proportion of SDS; any alteration in this respect may affect considerably the electromigration properties of the protein in a gel matrix. It has been demonstrated that at low gel concentrations (below 7.5%) considerably higher molecular masses than the real ones are observed with glycoproteins (for review see Ref. [14]). Such effects can be expected with glycated proteins as well.

4. Conclusions

Capillary gel electrophoresis using untreated capillaries filled with 4% non-cross-linked polyacrylamide was shown to be a suitable method for separating collagen type I polypeptide α-chains, their chain polymers (β_{11}, β_{12}, γ) and even appears suited for monitoring higher than γ-chain polymers. The method also was able to reveal differences in the electrophoretic pattern of collagens incubated with glucose. The differences were ascribed to both monotopic binding of glucose to the collagen α-chains and glucose-derived cross-link formation. Further investigations in this respect are necessary to confirm this hypothesis.

Acknowledgement

References

[1] K.I. Kivirikko, Ann. Med., 25 (1993) 113.
[2] E. Chung and E.J. Miller, Science, 183 (1974) 1200.
[3] P.P. Fietzek and K. Kühn, in D.A. Hall and D.S. Jackson (Editors), Int. Rev. Conn. Tiss. Res., Academic Press, New York, 1976, pp. 1–60.

[4] T. Hayashi and Y. Nagai, *J. Biochem. (Tokyo)*, 86 (1979) 453.

[5] J.W. Jorgenson and K.D. Lukacs, *Anal. Chem.*, 53 (1981) 1298.

[6] Z. Deyl, V. Rohlicek and M. Adam, *J. Chromatogr.*, 480 (1989) 371.

[7] H.F. Yin, J.A. Lux and G. Schomburg, *J. High Resolut. Chromatogr.*, 13 (1990) 624.

[8] D. Wu and F. Regnier, *J. Chromatogr.*, 608 (1992) 349.

[9] Z. Deyl and M. Adam, *J. Chromatogr.*, 488 (1989) 161.

[10] K.A. Smolenski, A. Fallon and N. Light, *J. Chromatogr.*, 287 (1984) 29.

[11] K.M. Reiser, *Proc. Soc. Exp. Biol. Med.*, 196 (1991) 17.

[12] K.M. Reiser, M.A. Amigable and J.A. Last, *J. Biol. Chem.*, 267 (1992) 24207.

[13] R.J. Butkowski, M.E. Noelken and B.G. Hudson, *Methods Enzymol.*, 82 (1982) 409.

[14] Z. Deyl, in Z. Deyl, F.N. Everaerts, Z. Prusík and P.J. Svendsen (Editors), *Electrophoresis—A Survey of Techniques and Applications, Part A: Techniques*, Elsevier, Amsterdam, 1979, pp. 53–58.