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Characterization of bovine collagens using capillary electrophoresis — an alternative to slab gel electrophoresis

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Abstract

A capillary electrophoresis method was developed and characterized for analyzing the spectrum of collagen subspecies in collagen preparations. The Bio-Rad CE-SDS protein kit was used for the dynamic sieving separation of collagen subspecies in this CE method (DSCE). The optimized method utilized a 36 cm (or 24 cm) × 50 μm uncoated capillary, electrophoretic injection at 10 kV for 10 s, a run voltage of 15 kV, a capillary temperature of 20°C, and UV detection at 220 nm. A preliminary validation of the method was performed. The assay had good repeatability (RSDs for peaks were 1–5%), and responses were linear for assay solutions with collagen concentrations from 0.125 to 1.25 mg/ml. The DSCE electropherogram of bovine skin collagen provided a profile of subspecies similar in number and relative abundance to that generated by scanning of Coomassie-stained SDS-PAGE gels.

Keywords: Capillary electrophoresis; Sodium dodecyl sulfate polyacrylamide gel electrophoresis; Bovine Type I collagen; Bovine Type III collagen

1. Introduction

Collagen constitutes the majority of the structural protein in such tissues as bone, skin, tendon, and cartilage, and is a prime component of the extracellular matrix of all tissues. The fibrillar collagen molecules (e.g. Types I, II, III, and V) are comprised of long triple helices comprising three intertwined primary chains. The three chains in the triple helix molecule, as well as neighboring helices in collagen fibrils, are held together through hydrogen bonds. There may also be one or more interstrand (intramolecular) covalent crosslinks in individual molecules [1], as well as helix-to-helix (intermolecular) crosslinks in fibrils. Under denaturing conditions (e.g. in the presence of sodium dodecyl sulfate, SDS) the hydrogen bonds between the helices or between the three chains of the triple helix break down and dissociate into individual units, as limited by the various covalent intra- and intermolecular crosslinks.

Conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been used for the identification, purity, and stability

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testing of collagen materials. On SDS-PAGE gels these collagen subspecies bands (α_1, α_2, β_{11}, β_{12}, γ, etc.) typically appear in a characteristic ladder-like array [1]. However, the SDS-PAGE technique is time-consuming and involves a multitude of steps, including running the gel, staining, destaining, and densitometry scanning for quantitation. The multiplicity of steps in this method often creates artifacts and causes variability. The linearity range of the band intensity on the gel is also narrow [2].

Capillary electrophoresis (CE) has been widely used in the separation and characterization of proteins and peptides. Size separation of proteins

Table 1
Determination of collagen subspecies by DSCE[a] and SDS-PAGE

Method	α_2 (%)	α_1 (%)	β_{12} (%)	β_{11} (%)	γ (%)	Total $>\gamma$ (%)
DSCE, 24 cm × 50 μm capillary (n = 6)						
Mean	10.5	37.6	30.3	4.2	8.5	9.0
S.D.	0.1	0.3	0.2	0.1	0.1	0.4
DSCE, 36 cm × 50 μm capillary (n = 6)						
Day 1 ($n = 4$)						
Mean	10.0	36.3	29.6	4.4	8.3	11.5
S.D.	0.2	0.4	0.3	0.5	0.1	0.4
Day 2 ($n = 2$)						
Mean	10.5	37.4	29.1	4.5	8.3	10.2
S.D.	0.3	0.2	0.2	0.4	0.1	0.7
SDS-PAGE (n = 11)						
Mean	10.1	30.8	24.2	3.3	17.3	14.2
S.D.	0.7	1.7	1.4	0.4	1.3	2.6

[a] Subspecies contents are presented as normalized time-adjusted peak area, which is (time-adjusted peak area/total time-adjusted peak area) × 100%. DSCE conditions: electrophoretic injection (10 kV/10 s), capillary cartridge temperature 20°C, and run voltage 15 kV.

Table 2
DSCE[a] system repeatability — electrophoretic injection vs. pressure injection

Collagen subspecies	α_2	α_1	β_{12}	β_{11}	γ	Total $>\gamma$
Electrophoretic injection[b] (n = 6)						
Migration time (min)						
Mean	10.40	11.12	12.50	12.94	14.41	–
S.D.	0.01	0.01	0.01	0.01	0.01	
Normalized peak area (% of total)						
Mean	10.5	37.6	30.3	4.2	8.5	9.0
S.D.	0.1	0.3	0.2	0.0	0.1	0.4
Pressure injection[c] (n = 6)						
Migration time (min)						
Mean	10.17	10.86	12.14	12.58	13.95	–
S.D.	0.01	0.01	0.02	0.02	0.02	
Normalized peak area (% of total)						
Mean	9.8	37.9	32.2	4.0	8.0	8.1
S.D.	0.3	0.4	0.5	0.2	0.1	0.3

[a] DSCE conditions: 24 cm × 50 μm uncoated capillary, capillary cartridge temperature 20°C, and run voltage 15 kV.
[b] Electrophoretic injection: 10 kV/10 s.
[c] Pressure injection: 60 psi × s.

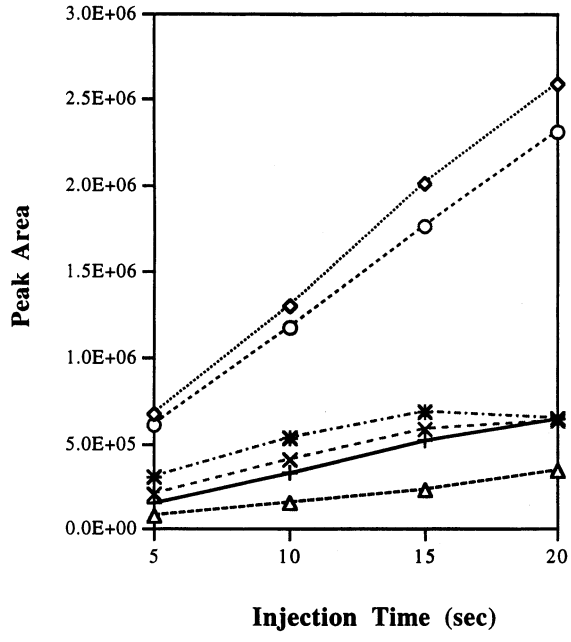

Fig. 1. Plot of peak area of collagen subspecies vs. electrophoretic injection time (+, α_2; ◇, α_1; ○, β_{12}; △, β_{11}; ×, γ; and *, $>\gamma$).

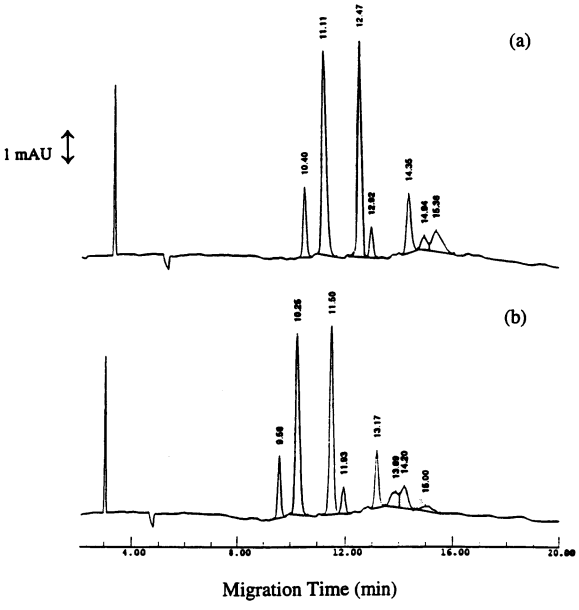

Fig. 2. Electropherograms of bovine skin collagen. DSCE conditions: uncoated 24 cm × 50 μm capillary, electrophoretic injection at 10 kV for 10 s, run voltage at 15 kV, and capillary temperature at (a) 20°C and (b) 25°C.

in gel-filled capillaries was first described by Hjerten [3] and later by Cohen and Karger [4]. The use of non-gel sieving CE, in which capillaries are filled with noncross-linked polymer modified solution, was reported by Chin and Colburn [5]. Several workers have published papers on the separation of collagen using gel-filled CE or non-gel sieving CE [6–9]. However, in those studies only qualitative analyses were reported. The quantitative assessment for the identity, purity, and stability of collagen using DSCE has not been extensively explored.

In this study the subspecies profile of bovine skin collagen was characterized and quantitated by non-gel dynamic sieving capillary electrophoresis (DSCE) and by SDS-PAGE. The possibility that DSCE could be used as an alternative to SDS-PAGE was evaluated.

2. Experimental

2.1. Materials

CE-SDS protein sample buffer, CE-SDS protein run buffer, CE-SDS protein size standard, and CE-SDS internal standard (benzoic acid), acrylamide, SDS, Tris, and dithiothreitol were purchased from Bio-Rad (Hercules, CA, USA).

Bovine skin collagen solution (pepsinized, containing approximately 90% Type I and 10% Type III collagen) was produced by Matrix Pharmaceutical (Fremont, CA, USA). Types I and III collagens were isolated and purified from this bovine collagen solution by salt fractionation [10]: Type III collagen was precipitated from collagen solution using dialysis against 50 mM Tris buffer (pH 7.15) containing 1.1 M NaCl, and Type I collagen was precipitated from the supernatant by the addition of 0.2 M sodium phosphate buffer (pH 10.7).

2.2. Instrumentation and methods

2.2.1. CE

A Bio-Rad BioFocus 3000 equipped with a multiple wavelength UV detector was used. Uncoated fused-silica capillaries with length of 24 or

36 cm (19.4 or 31.4 cm to the detector) and ID of 25, 50, 75 or 100 μm mounted onto a Bio-Rad self-assemble cartridge, were purchased from J&W Scientific (Folsom, CA, USA). A polyvinylalcohol-coated capillary purchased from Hewlett-Packard (Santa Clara, CA, USA) was used for comparison. Analyte peak areas were normalized to migration times to compensate for the different migrating speeds through the detector.

2.2.2. SDS-PAGE

Collagen samples were denatured in SDS, then run on 4–15% gradient PAGE gels and stained with Coomassie Blue or run on 4–20% gradient PAGE gels and stained with silver. The relative amounts of the collagen subspecies in the Coomassie-stained gels were quantitated with a densitometer (personal densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA). A gel eluter (SixPac, Hoefer Scientific Instrument, USA) was used for isolating the collagen subspecies from unstained gels.

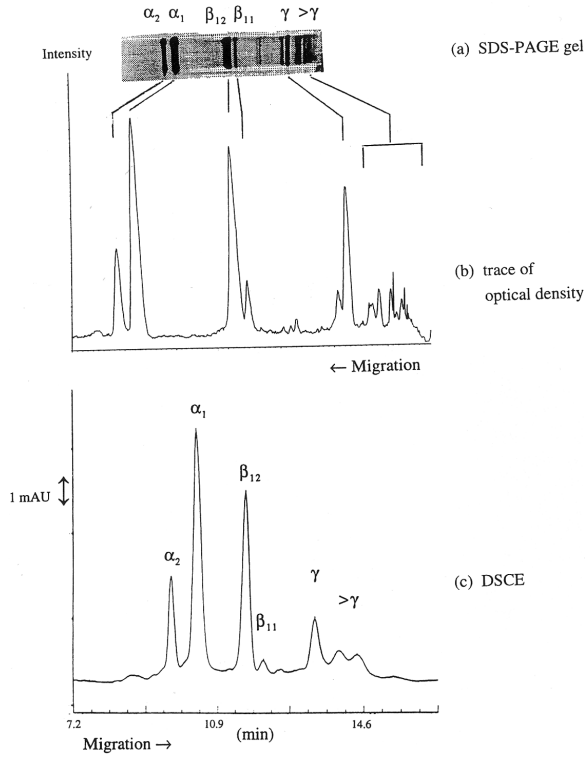

Fig. 3. (a) Coomassie blue-stained SDS-PAGE gel, (b) optical density trace of SDS-PAGE and (c) DSCE of bovine skin collagen.

2.3. Method development: optimization for DSCE conditions

Using a generic method developed for protein analysis by Bio-Rad Laboratories [11] as the point of departure, experiments were performed to optimize both sample preparation and DSCE operating parameters for the analysis of collagen samples. These included the evaluation of different capillaries, injection modes, injection times, capillary temperatures, and run voltages.

2.3.1. Capillary

Although the resolution for the CE separation can often be improved by increasing the capillary length and decreasing the capillary internal diameter, there are trade-offs in the runtime, run voltage, and sensitivity. In this study uncoated fused-silica capillaries with 25, 50, 75 or 100 μm ID, and 24 or 36 cm length were evaluated. Both 24 and 36 cm × 50 μm capillaries provided reasonable resolutions, sensitivities, and runtimes for the characterization of collagen subspecies. The 36 cm × 50 μm capillary provided better resolution but took a longer runtime (40 min) compared to the 24 cm × 50 μm capillary (20 min). The 24-cm and 36-cm capillaries provided equivalent results (Table 1). A polyvinylalcohol-coated capillary was also examined. The migration patterns and migration times of bovine skin collagen in coated and uncoated capillaries were very similar (electropherograms not shown). Since the pH of the electrophoresis buffer is high (ca. 8.9) and the denatured collagen/SDS complex forms a charged micelle-like species, electroosmosis and protein adsorption effects are not significant in this system [12], and a coated capillary is not needed.

2.3.2. Injection mode

Both electrophoretic and pressure injections were evaluated. In theory, during electrophoretic injection sample ions migrate into the capillary in proportion to their electrophoretic mobilities. Consequently, electrophoretic injection may be

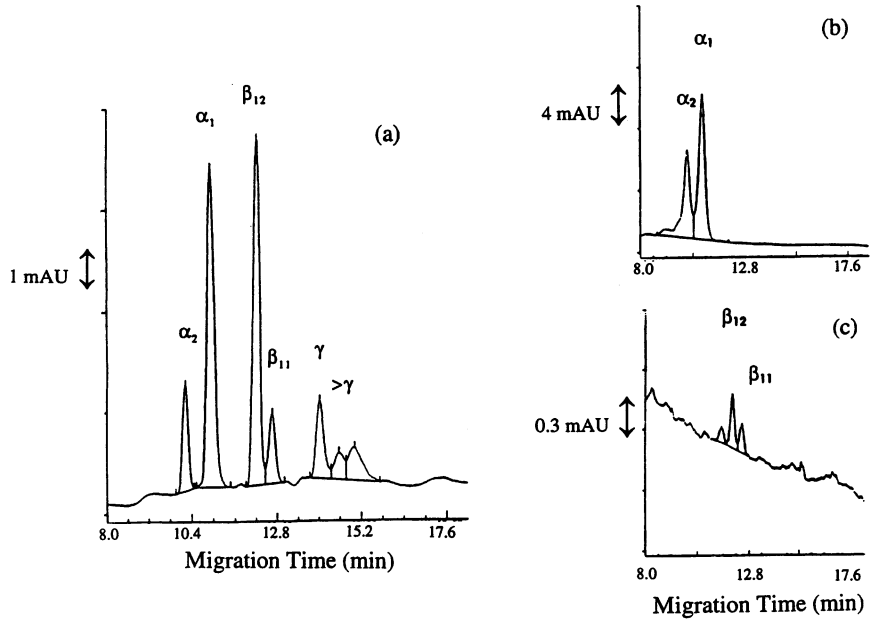

Fig. 4. DSCE electropherograms of α and β fractions collected from SDS-PAGE gel. (a) Collagen; (b) α bands from SDS-PAGE gel; and (c) β bands from SDS-PAGE gel.

component selective and may introduce a sample bias. Pressure injection produces a sample zone with analytes at the same concentrations as in the sample solution.

For bovine skin collagen material, the electropherograms and the resultant contents of collagen subspecies obtained from electrophoretic injection (10 kV for 10 s) and pressure injection (60 psi × s) were compared (Table 2). The relative peak areas seen after electrophoretic injection were essentially equivalent to those obtained after pressure injection, showing that component selectivity was not significant during electrophoretic injection. However, the system precision obtained from electrophoretic injection was slightly better (i.e. with lower relative standard deviation, R.S.D.) than that from pressure injection (Table 2). In addition, electrophoretic injection produced a better baseline and a higher sensitivity (signal-to-noise ratio) than pressure injection (data not shown).

2.3.3. Injection time

Electrophoretic injection with various injection times between 5 and 25 s was evaluated. A collagen concentration of 0.75 mg/ml and an injection voltage of 10 kV were used throughout. The peak areas showed good linearity for α_2, α_1, β_{12}, and β_{11} peaks for the injection range from 5 to 20 s, whereas the peak areas for γ and $>\gamma$ peaks were linear only up to 15 s. Above 15 s there was poor resolution between γ and $>\gamma$ peaks. Based on this study, an optimal electrophoretic injection time of 10 s was selected (Fig. 1).

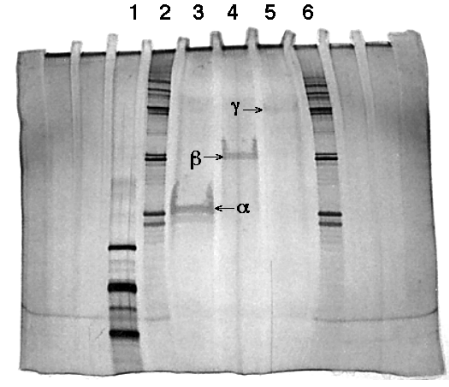

Fig. 5. Silver-stained SDS-PAGE gel of α, β and γ fractions collected from DSCE. (lane 1, protein standards; lane 2 and lane 6, bovine skin collagen)

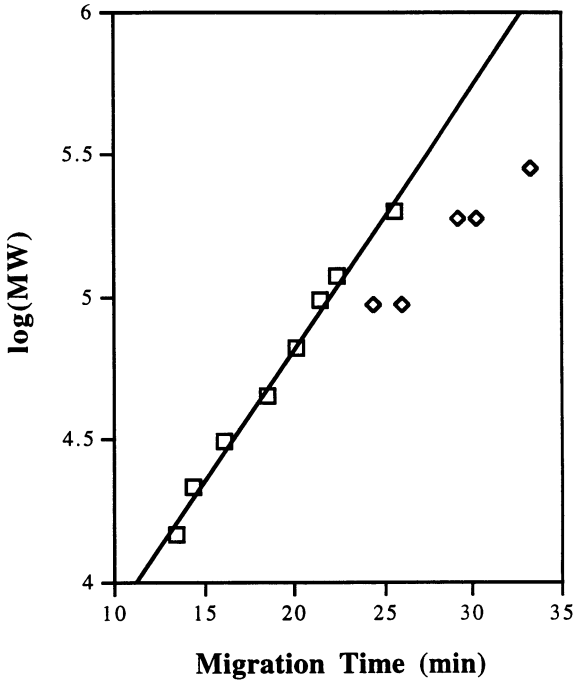

Fig. 6. A plot of log MW vs. migration time from DSCE electropherograms of protein standards and collagen subspecies (□, protein standards and ◇, collagen subspecies).

2.3.4. Capillary temperature/run voltage

Raising the capillary temperature or run voltage should decrease the viscosity of the run buffer and increase the electrophoretic mobility of the analyte. It may result in shorter migration times but lower resolutions for the analytes [13]. Bovine skin collagen material was electrophoresed at various capillary temperatures (i.e. 15, 20 and 25°C) and run voltages (i.e. 15 and 20 kV). Some higher molecular weight peaks (γ and $>\gamma$) were lost when the samples were electrophoresed at 15°C, probably because of higher viscosity of the run buffer. Satisfactory results were obtained when the samples were electrophoresed at 20 or 25°C (Fig. 2). The electropherogram run with 15 kV at 20°C provided the best resolution and baseline with a reasonable runtime.

The proposed DSCE operating conditions for the characterization of bovine collagen are therefore as follows: capillary, 24 cm (or 36 cm) × 50 μm ID uncoated fused-silica capillary; polarity, negative to positive; buffer, CE SDS-protein run

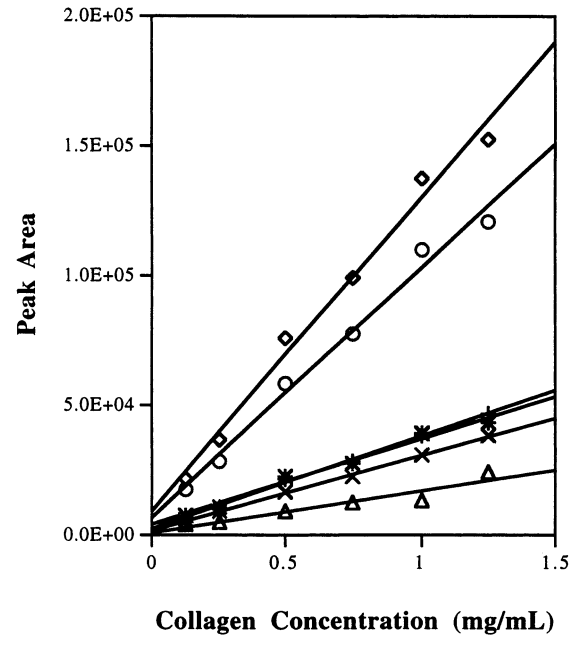

Fig. 7. Linearity of collagen subspecies separated by DSCE. (+, α_2; ◇, α_1; ○, β_{12}; △, β_{11}; ×, γ; and *, $>\gamma$).

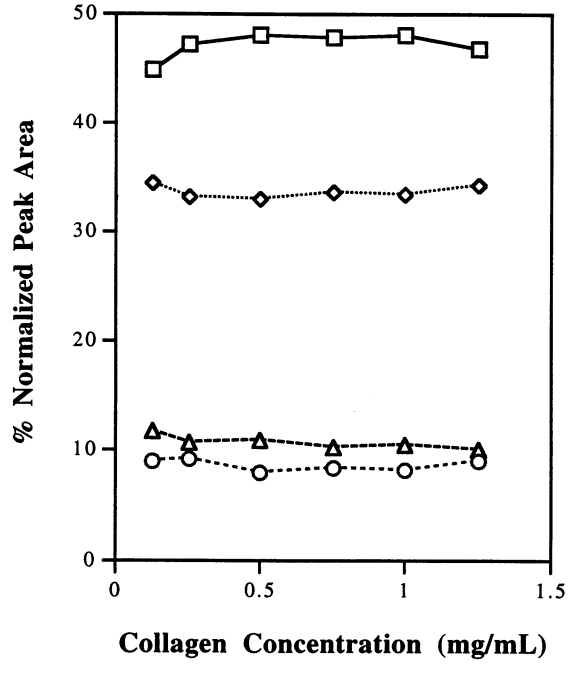

Fig. 8. A plot of percent normalized peak area of collagen subspecies vs. collagen concentration (□, $\alpha_2+\alpha_1$; ◇, $\beta_{12}+\beta_{11}$; △, $>\gamma$; and ○, γ).

Table 3
Comparison of DSCE to SDS-PAGE in the characterization of collagen

	DSCE	SDS-PAGE
Total analysis time	0.5–1.5 days	2–3 days
Preparation	Assemble capillary cartridge, prepare SDS-protein samples, load samples and reagents into carousels, program the software	Pour gel, prepare SDS-protein samples, prepare run buffer, load samples
Procedure	Run capillary electrophoresis	Run gel electrophoresis, fixation, Coomassie blue staining, destaining
Analysis	Analyze electronic data	Scan gel by densitometer, draw grids manually, perform integration
Run buffer needed	0.5–2 ml	5–10 l
Waste generated	2–10 ml	5–15 l
Maximum numbers of samples	22 samples per carousel	12 samples per gel
Automation	Yes	No
On-line detection	Direct UV detection	No

buffer; electrophoretic injection, 10 kV for 10 s; run voltage, 15 kV; detection, 220 nm; cartridge temperature, 20°C; and the runtime, about 20 min (ca. 40 min if using a 36 cm × 50 μm capillary).

2.4. DSCE sample preparation

A collagen solution (200 μl) in 10 mM HCl was mixed with 200 μl Bio-Rad CE-SDS sample buffer and 10 μl internal standard. The mixture solution was incubated in a 90–100°C water bath for 10 min. The sample solution was cooled to room temperature and centrifuged at 6000 rpm for 2 min prior to injection. For reduced sample preparations, dithiothreitol solution was added to a final concentration of 2.5%.

3. Results and discussion

3.1. Characterization of bovine skin collagen

Bovine skin collagen samples were prepared and electrophoresed by SDS-PAGE and DSCE methods. The SDS-PAGE gel banding pattern in the sequence of α_2, α_1, β_{12}, β_{11}, γ, $>\gamma$ (Fig. 3), was similar to that in the literature [1]. The optical density trace of the collagen subspecies from the densitometer scan of Coomassie-stained SDS-PAGE gel and the electropherogram of the same lot of collagen from DSCE were similar in terms of both peak spacing and relative peak areas. It is of note that due to the different separation mechanisms the migration directions of the collagen subspecies on the SDS-PAGE gel and DSCE electropherogram are reversed.

In order to identify the collagen peaks in the DSCE, the collagen subspecies separated on SDS-PAGE were eluted from the gel and electrophoresed on DSCE. The migration times of the first four bands (putatively identified as α_2, α_1, β_{12} and β_{11}) well matched those of the first four peaks of collagen on the electropherogram. The relative peak areas were also consistent with the peak identities (Fig. 4).

Fractions from the DSCE separation of collagen were also collected and verified by SDS-PAGE. Fraction 1 contained the first two peaks, fraction 2 contained the third and fourth peaks, and fraction 3 contained all the other peaks. These fractions were run on SDS-PAGE and the

protein bands were visualized by silver staining. Using the collagen reference material, the fractions from DSCE were positively identified as the α bands, β bands and γ bands (Fig. 5).

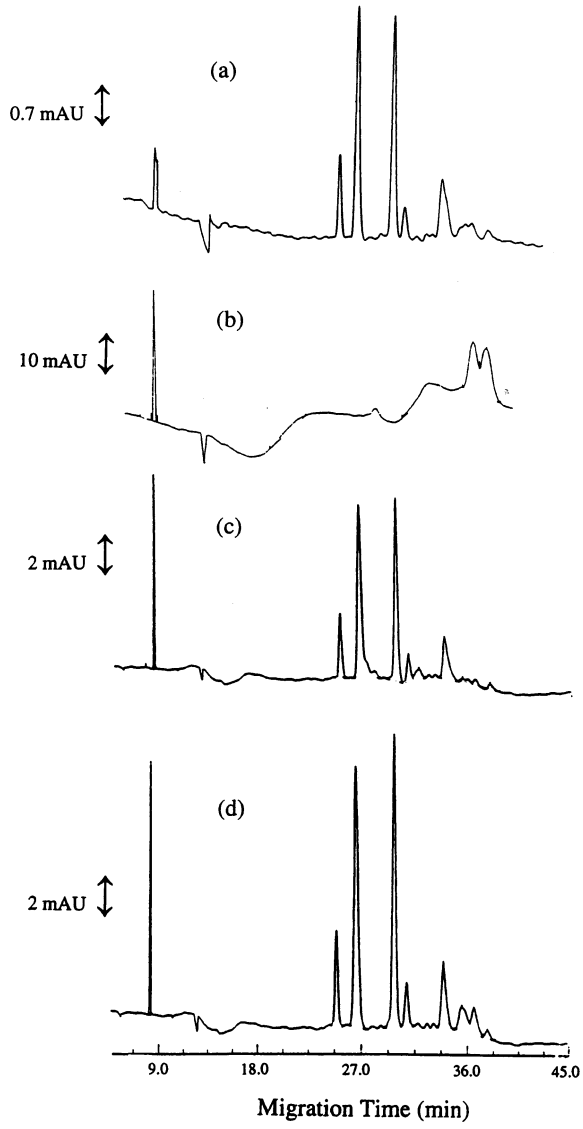

Fig. 9. DSCE electropherograms of (a) a nonreduced pure Type I collagen, (b) a nonreduced Type III collagen, (c) a reduced bovine skin collagen, and (d) a nonreduced bovine skin collagen. DSCE conditions: uncoated 36 cm × 50 μm capillary, electrophoretic injection at 10 kV for 40 s, run voltage at 15 kV, and capillary temperature at 20°C.

3.2. Anomalous migration times for bovine skin collagen subspecies

A plot of log MW versus migration time from a typical electropherogram of CE-SDS protein standards (Bio-Rad) is shown in Fig. 6. This standard solution contains lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), serum albumin (66.2 kDa), phosphorylase B (97 kDa), β-galactosidase (116 kDa), and myosin (200 kDa). The molecular weights of α_2, α_1, β_{12}, β_{11} and γ collagen subspecies based on the primary sequence are 93, 93, 186, 186 and 279 kDa [1]. The log MW versus migration time plot of the collagen subspecies did not fall onto the standard curve (Fig. 6). The apparent discrepancy in the migration behavior of the collagen subspecies may be due to differences in SDS binding stoichiometry between collagen and the CE-SDS protein size standards and/or differences in shape of the denatured mass. Collagen differs significantly from other proteins in its amino acid composition and amino acid sequence [1]. Collagen is high in glycine and proline, and contains the rare amino acids 4-hydroxyproline and 5-hydroxylysine. In addition, the proline content may make the collagen–SDS complex more rigid than other protein–SDS complexes, thus increasing the hydrodynamic radius of the collagen–SDS complex. It is of note in this regard that the collagen subspecies showed higher apparent molecular weights by SDS-PAGE.

3.3. System performance

3.3.1. Precision

Experiments were performed using three sample vials. Two electrophoretic injections (10 kV for 10 s) were taken from each sample vial and injected onto a 24 cm × 50 μm uncoated capillary. The RSDs for the migration times of each subspecies were only in the range 0.1–0.2%. The RSDs for the time-adjusted peak area percents (Table 2) were less than 5%.

3.3.2. Linearity and range

Linearity was examined using Matrix bovine collagen at concentrations of 0.125–1.25 mg/ml.

Each sample was injected in duplicate. Good linearity was demonstrated for α_2, α_1, β_{12}, β_{11}, γ, and $>\gamma$ peak areas for the whole concentration range studied (Fig. 7). The r^2 values for α_2, α_1, β_{12}, β_{11}, γ, and $>\gamma$ peak areas were 0.996, 0.987, 0.988, 0.914, 0.998 and 0.985, respectively. The above experiments show that the peak responses are linear with respect to collagen concentration over a 10-fold range from 0.125 to 1.25 mg/ml.

The time-adjusted peak area percents for the α, β, γ and $>\gamma$ peaks (Fig. 8) were most consistent over the collagen concentration range from 0.25 to 1.0 mg/ml, with RSDs of 3.3, 3.1, 5.9 and 9.7%, respectively, for the four peaks.

3.4. Comparison of DSCE to SDS-PAGE analysis for bovine skin collagen

Quantitative results from the analysis of bovine skin collagen by DSCE and SDS-PAGE are summarized in Table 1. The DSCE data were taken from the system precision experiment and the SDS-PAGE data were taken from multiple gels of a collagen material. Compared to the SDS-PAGE data, DSCE peak area percents were slightly higher for α and β peaks and slightly lower for γ and $>\gamma$ peaks. The differences between the SDS-PAGE and DSCE data may reflect differences in Coomassie blue staining among the different collagen subspecies in SDS-PAGE or differences in UV absorptivities of the various subspecies in DSCE. The repeatability of the DSCE assay was better than that of the SDS-PAGE assay. In general the DSCE method provided better resolutions between α_2 and α_1, and β_{12} and β_{11} peaks than did SDS-PAGE gels; on the other hand, the resolution between the γ and $>\gamma$ peaks with DSCE was not as good as on the SDS-PAGE gels (Fig. 3).

The advantages and disadvantages of DSCE and SDS-PAGE methods in bovine skin collagen analysis are compared in Table 3. Compared to SDS-PAGE, DSCE has several advantages. These include a shorter runtime, smaller volumes of run buffers needed, in-line UV detection, automation and reduction in waste. In the collagen assay DSCE provides better resolutions between α_2 and α_1 and between β_{12} and β_{11} peaks. The disadvantages of this DSCE method are, besides the higher cost of the equipment, the lower resolution between γ and $>\gamma$ peaks and the lower sensitivity (signal/noise). The latter, however, can be improved using a bubble-cell capillary, a Z-shaped capillary, or other techniques [14].

3.5. Bovine Types I and III collagens

The bovine Type III collagen triple helix is disulfide-bridged. Under nonreduced conditions the inter-chain disulfide bonds are intact. It is expected, then, that the $[\alpha_1(III)]_3$ chains should have mobility on SDS-PAGE similar to that of the γ(I) and the $>\gamma$(I) bands. Under reducing conditions, in contrast, the disulfide bonds are cleaved and the α_1(III) monomers should migrate similarly to the α_1(I) chains.

Pure Type I and Type III bovine collagens isolated from bovine skin collagen were analyzed by DSCE in nonreduced form (Fig. 9a and b). Bovine skin collagen samples (containing about 90% Type I and 10% Type III collagen) in reduced and nonreduced sample buffers were also prepared and analyzed by DSCE (Fig. 9c and d). By comparing the electropherogram of nonreduced collagen (Fig. 9d) to that of the nonreduced Type III collagen (Fig. 9b) it is clear that the $>\gamma$ peaks in the nonreduced collagen sample contain Type III collagen. This conclusion was further confirmed using the reduced collagen samples. Compared to the nonreduced sample the reduced sample (Fig. 9c) showed a decrease in the $>\gamma$ peaks and the appearance of two new peaks, one immediately following the α_1(I) peak and one after the β_{11}(I) peak. This experiment suggests that it would be possible also to quantitate Type III bovine collagen in collagen samples by DSCE.

4. Conclusion

This study shows that the DSCE electropherogram of bovine skin collagen was very similar in number and relative abundance of subspecies to the profile generated by SDS-PAGE. The identity of the banding pattern was established. SDS-PAGE is selective but is labor-intensive and oper-

ator-dependent. The DSCE method is more reproducible, simple, rapid, and automated and can separate collagen subspecies to the same banding pattern as SDS-PAGE. Therefore, the DSCE method may provide an attractive alternative to SDS-PAGE as a routine method for quality control and stability testing of relative subspecies distribution for bovine collagen materials.

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