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Journal of Chromatography A, 1008 (2003) 233–245
JOURNAL OF CHROMATOGRAPHY A
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Capillary electrochromatographic separation of bovine milk proteins using a G-quartet DNA stationary phase

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Received 28 October 2002; received in revised form 22 April 2003; accepted 27 May 2003

Abstract

DNA oligonucleotides that form G-quartet structures were used as stationary phase reagents for separation of bovine milk proteins, including α-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin. Both artificial protein mixtures and a skim milk sample were analyzed. The separations were performed using open-tubular capillary electrochromatography, in which the oligonucleotides were covalently attached to the inner surface of a fused-silica capillary. Better resolution was achieved using the G-quartet-coated capillaries than was achieved using either a bare capillary or a capillary coated with an oligonucleotide that does not form a G-quartet structure. A 4-plane G-quartet-forming stationary phase was able to resolve three peaks for α-casein and to detect thermal denaturation of the proteins in the milk sample. The results suggest that G-quartet stationary phases could be used to separate very similar protein structures, such as those arising from genetic variations or post-translational modifications.
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Keywords: Stationary phases, CEG; Electrochromatography; Bovine milk proteins; DNA

1. Introduction

The protein fraction of bovine milk is divided into two classes based on differing solubility behaviors at pH 4.6: the caseins and the whey, or serum, proteins. The caseins are a family of phosphoproteins that aggregate to produce the micellar properties of milk. They are insoluble at their isoelectric point (pH 4.6 and 20 °C) at make up 80% of the milk protein fraction. The casein fraction is comprised of α_{s1}-casein (α_{s1}CN), α_{s2}-casein (α_{s2}CN), β-casein (βCN), and κ-casein (κCN). The whey proteins are soluble at pH 4.6 and make up the remaining 20% of the milk protein fraction. α-Lactalbumin (αLa) and β-lactoglobulin (βLg) are the most prevalent proteins in whey, which also contains bovine serum albumin, immunoglobulin G, lacto(trans)ferrin, and other minor proteins. The diversity of bovine milk proteins is further complicated by genetic polymorphism (as many as seven variants have been observed for a single protein, and the presence of each varies among different cattle breeds [1–3]) and by post-translational modifications.

Bovine milk proteins offer considerable nutritional value for humans. There has been an increasing interest in their use as additives in formulated foods and for purposes such as nutritional supplements, nutraceuticals, flavorings, and bioactive ingredients

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doi:10.1016/S0021-9673(03)00973-7

[4–6]. Improved technology is necessary for the selective isolation of a particular protein to be used as a food additive, as well as for the process control and quality control of the new products. For some individuals, particularly infants, selective removal of certain allergenic proteins from bovine milk would be beneficial [7,8]. Improved technology is also necessary for the evaluation of heat treatment of milk, since excessive heating may cause instability or gelling in the manufacturing processes of some milk products [9]. Detection of denatured whey proteins is believed to be a good indicator of heat-damaged milk [10]. Reasons such as these have intensified the demand for dependable, efficient techniques for non-denaturing separation and analysis of bovine milk proteins.

Traditional techniques for the separation of bovine milk proteins include reversed-phase HPLC [4,8,11,12], ion-exchange chromatography [13–15], perfusion chromatography [16], gel electrophoresis [17,18], isoelectric focusing [19,20], and more recently, capillary electrophoresis (CE) [2,10,21–25]. While each of these methods has its own merits, they suffer from a number of limitations. In RP-HPLC, protein adsorption to column and support surfaces is common, resulting in poor separation efficiency and low protein recovery. Ion-exchange chromatography, gel electrophoresis, and isoelectric focusing are time-consuming and labor-intensive. Furthermore, the above-mentioned techniques often result in protein denaturation, which is undesirable in some analytical applications as well as preparative work.

CE is an attractive alternative for analysis of milk proteins, offering rapid, automated separations with on-line detection and requiring only minute sample and buffer volumes. Currently, CE is the predominant method used to separate the bovine whey proteins [23,25–30]. However, a common problem encountered in CE is protein adsorption to the capillary surfaces, which leads to band broadening, unstable electroosmotic flow, and a reduction in separation efficiency [31,32].

Capillary electrochromatography (CEC) [33,34] is a hybrid technique that combines the strategies of both HPLC and CE. As in HPLC, the proteins partition between mobile and stationary phases, but rather than the parabolic, laminar flow profile obtained from a pressure gradient in HPLC, flow in CEC is driven by electroosmosis. This results in a flat, plug-like flow profile, yielding more efficient separations in much less time. The use of a capillary also greatly reduces the sample and buffer volume requirements relative to HPLC.

This paper describes the use of G-quartet-forming DNA oligonucleotides as novel stationary phases in open-tubular CEC (OTCEC) for the separation of bovine milk proteins. The oligonucleotides used in this work include 2-plane and 4-plane G-quartet structures. The 2-plane structure is formed by the thrombin-binding aptamer [35–37]. The 4-plane structure has the same sequence as the thrombin-binding aptamer [35–37], with the exception of the number of G-repeats that form the G-tetrad planes. The two structures are shown in Fig. 1. Unlike the 2-plane aptamer, the 4-plane G-quartet forms in the absence of cations such as K^+ and is unusually stable. The melting temperature (T_m) that is associated with the unfolding of the 4-plane G-quartet structure is 28 °C in the absence of K^+ [38] and >70 °C in the presence of 1 m*M* K^+.

In a previous study, we demonstrated the potential of the 4-plane G-quartet stationary phase in OTCEC to separate the two most common genetic variants of bovine βLg [39], variants A (βLgA) and B (βLgB). The variants differ by only two amino acid residues: variant A (βLgA) has Val118, Asp64, and variant B (βLgB) has Ala118, Gly64 [40]. The results indicated that the G-quartet conformation plays a role in the separation of the two proteins. The present work extends the investigation of G-quartet stationary phases to the separation of both whey and casein proteins in mixtures of purified proteins and in bovine skim milk. The focus is on the benefits of the G-quartet structure for analysis of the complex protein mixtures relative to CZE on a bare capillary or CEC using an oligonucleotide that has the same base composition as the 2-plane aptamer but in a different sequence that does not form a G-quartet structure.

2. Experimental

2.1. Materials

Bovine αLa, βLgA, βLgB, αCN, βCN, and κCN were obtained from Sigma (St. Louis, MO, USA). αLa, αCN, βCN, and κCN were stored at −20 °C,

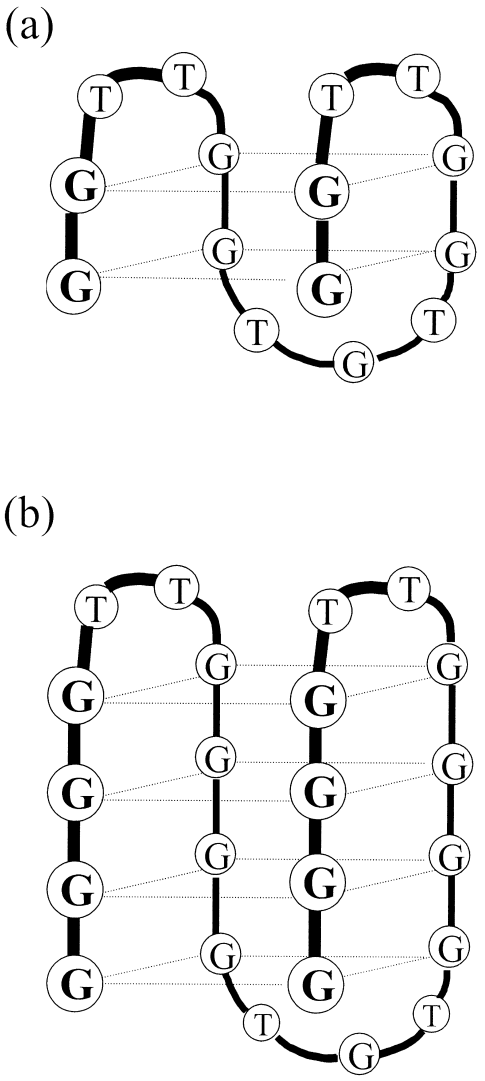

Fig. 1. (a) G-quartet structure of the 2-plane, thrombin binding aptamer and, by inference, (b) of the 4-plane oligonucleotide.

and βLgA and βLgB were stored at 4 °C. Skim milk was obtained from a local grocery store and stored at 4 °C. Upon use, the milk was diluted to 25% in the mobile phase buffer (10 m*M* phosphate, pH 7.3) and filtered through a 0.45-μm acetate filter. No further treatment was performed. Each sample was used before the 'sell by' date.

The 5′-thiol-modified oligonucleotides, including the 2-plane G-quartet-forming oligonucleotide (5′-(RSSC)-GGTTGGTGTGGTTGG-3′), which shall be referred to as **1**, the 4-plane G-quartet-forming oligonucleotide (5′-(RSSC)-GGGGTTGGGGTGTG-GGGTTGGGG-3′), which shall be referred to as **2**, and the 'scrambled' sequence oligonucleotide (5′-(RSSC)-GGTGGTGGTTGTGGT-3′), which shall be referred to as **3**, were custom-synthesized by Midland Certified (Midland, TX, USA) and used as received. Stock solutions of the oligonucleotides (2.5 m*M*) were prepared in water and stored at −20 °C. Sodium phosphate and (3-aminopropyl)triethoxysilane were obtained from Sigma. Sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate and tris(2-carboxyethyl)phosphine were obtained from Pierce Chemical (Rockford, IL, USA). HPLC-grade solvents were used in all solution preparations.

2.2. Capillary preparation

All capillaries were prepared using fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA), 75-μm inner diameter, unless otherwise indicated, 360-μm outer diameter, and 47 cm in length. Each capillary was first activated by incubation at room temperature with methanol for 10 min, water for 2 min, 1.0 *M* NaOH for 10 min, and water for 2 min. For experiments using bare capillaries, the capillaries were used with no further treatment. For the oligonucleotide-coated capillaries, the oligonucleotides were covalently attached to the activated inner capillary surface using an organic linker molecule, sulfosuccinimidyl 4-(maleimidolmethyl) cyclohexane-1-carboxylate (S-SMCC), by the method of Phillips and Chmielinska [41]. A 1 m*M* solution of the thiol-modified oligonucleotide was incubated with tris(2-carboxyethyl)-phospine (TCEP) prior to introduction into the capillary to reduce the 5′-disulfide to a free thiol. The modified oligonucleotide was then coupled to the linker on the capillary surface by the method of O'Donnell et al. [42]. The oligonucleotide-coated capillaries were rinsed with 100 m*M* phosphate buffer (pH 7.4) and stored at 4 °C.

2.3. CEC

Capillaries were mounted in temperature-controllable CE cartridges from Beckman (Palo Alto, CA, USA). Each capillary was used for approximately 20–50 runs and stored at 4 °C when not in use. CEC

separations were performed using a Beckman P/ACE 5000 CE system with UV absorption detection at 280 nm. Sodium phosphate buffer (10 m*M*, pH 7.3) served as the mobile phase. Protein samples were introduced into the capillary using low-pressure injection for 5 s and the EOF was driven by a 15-kV applied potential, unless otherwise noted, in forward polarity. Separations were performed at 25 °C.

2.4. Heat denaturation experiments

A 25% skim milk sample and solutions of the individual, commercial milk proteins were used for the heat denaturation experiments. Samples were incubated at 90 °C for 25 min in a vacuum oven. The samples were allowed to cool to room temperature, and CEC separations were performed as described above.

3. Results and discussion

3.1. Separations of mixtures of purified proteins

OTCEC results using a **2**-coated capillary for separation of a mixture of βLgA, βLgB, αCN, βCN and κCN are shown in Fig. 2a. The peaks were identified by independently spiking the mixture with each of the proteins. The elution order was determined to be βCN, βLgB, βLgA, αCN, κCN. Three peaks were identified for αCN and baseline separation of βLgA, βLgB, and βCN was achieved. αCN and κCN gave broad peaks with long retention times, and κCN was only partially resolved from the third, broad peak of αCN.

As shown in Fig. 2b, CEC of the same mixture with a **3**-coated capillary does not provide any separation of the αCN and κCN proteins, nor are the other proteins well resolved.

The third major bovine whey protein (αLa) was added to a mixture of the other whey components (βLgA and βLgB) and βCN. Separation of these four proteins was achieved using the **2**-capillary coated capillary (Fig. 2c). αLa eluted first, but was only partially resolved from βCN (R=0.97).

Fig. 2d shows a mixture of αLa, βLgA, βLgB, αCN, βCN, and κCN on a narrower **2**-coated capillary (25 μm instead of 75 μm I.D.), lower protein concentrations, and absorption detection at 214 nm instead of 280 nm to improve detectability for the shorter pathlength. As expected, the peaks are narrower and elution is faster than on the wider capillary. However, resolution is not improved, and is actually worse for β-CN, which is not detected as a separate peak in the mixture, as it was using the larger inner diameter capillary. A possible explanation is that the slowly diffusing proteins, upon reaching the stationary phase at the capillary surface, are more likely to encounter another interaction site as they are driven forward by the EOF than to leave the vicinity of the stationary phase and diffuse back into the bulk mobile phase. Thus, the effective ratio of stationary phase to mobile phase that is experienced by the proteins is greater than would be predicted based on the bulk mobile phase volume, and the expected increase in resolution with decreasing capillary diameter is not observed.

Results for individual runs of the casein proteins on **1**-coated, **2**-coated, and bare capillaries are shown in Fig. 3. The elution order of the individual caseins on the **1**- and **2**-coated capillaries is the same as for the caseins in the mixture: βCN, αCN, κCN. The bare capillary gave a different elution order that varied over the lifetime of the capillary. Presumably, the bare capillary is coated over time with the various proteins, changing both the EOF and the interactions of the proteins with the capillary surface.

Fig. 3 also shows the results for αCN run on the **3**-coated capillary. It is likely that the three peaks observed for αCN on the **2**-coated capillary arise from the presence of three αCN proteins in the commercial preparation, including α_{s2}CN and the two genetic variants of α_{s1}CN (α_{s1}CN-B and α_{s1}CN-C). The three peaks are best resolved using the **2**-coated capillary and only slightly indicated using the **1**-coated capillary. Neither the **3**-coated (non-G-quartet) capillary nor the bare capillary shows any evidence of multiple peaks for the α-casein. The poor resolution of the three proteins using the **1**-coated capillary is attributed to the absence of stabilizing K^+ in the mobile phase, which minimizes formation of the 2-plane G-quartet structure by **1**. The absence of K^+ has much less impact on the more stable, 4-plane structure of **2**.

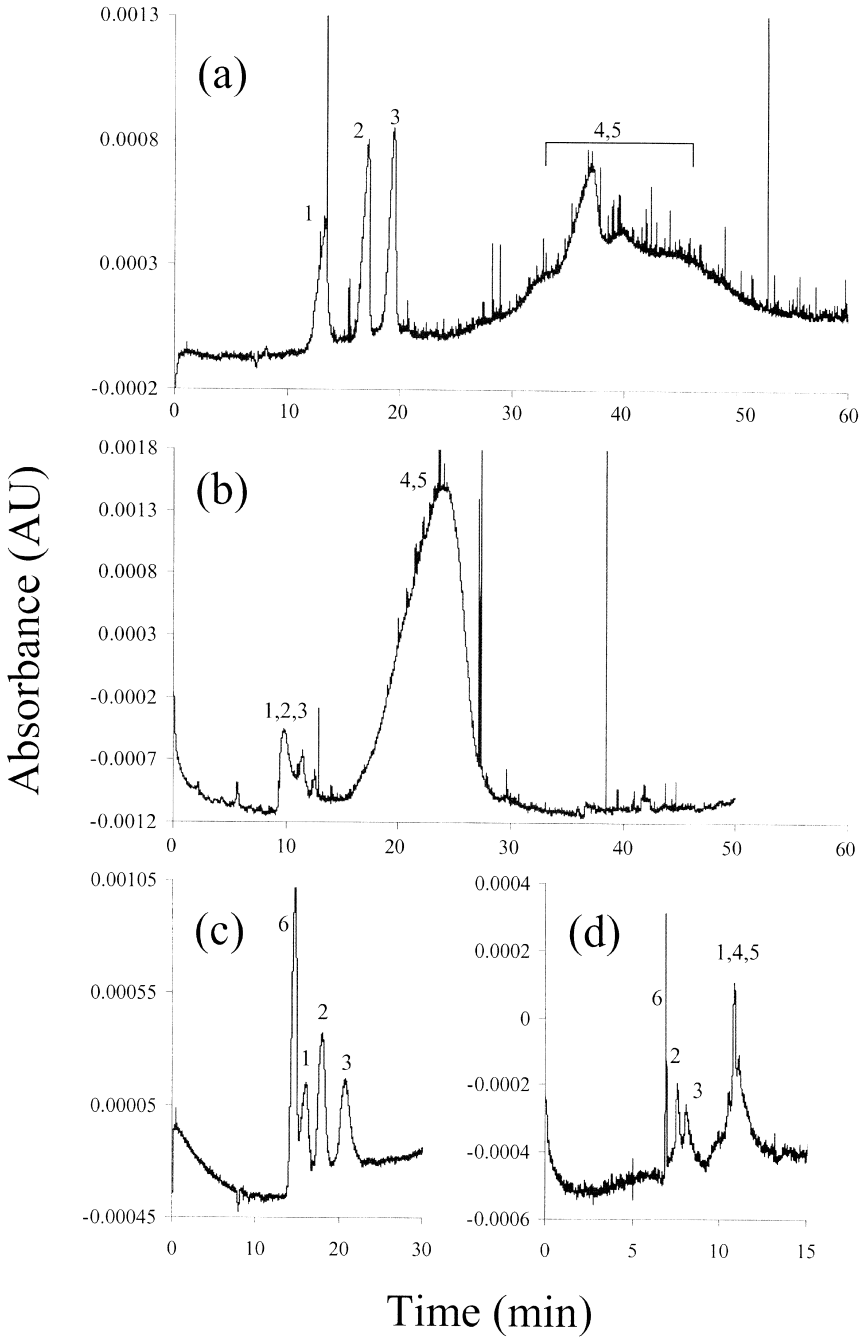

Fig. 2. Results for mixtures obtained using (a) the **2**-coated capillary (75 μm I.D., EOF=7.0 min), (b) the **3**-coated capillary (75 μm I.D., EOF=6.0 min), (c) the **2**-coated capillary (75 μm I.D., EOF=7.0 min), and (d) the **2**-coated capillary (25 μm I.D., EOF=4.8 min). Mixtures were 25 μ*M* βLgA, 25 μ*M* βLgB, 63 μ*M* αCN, 63 μ*M* βCN, and 63 μ*M* κCN for (a) and (b); 25 μ*M* αLa, 25 μ*M* βLgA, 25 μ*M* βLgB, and 63 μ*M* βCN for (c); and 20 μ*M* αLa, 20 μ*M* βLgA, 20 μ*M* βLgB, 50 μ*M* αCN, 50 μ*M* βCN, and 50 μ*M* κCN for (d). Peaks: 1=βCN; 2=βLgB; 3=βLgA; 4=αCN; 5=κCN; 6=αLa. Mobile phase: 10 m*M* phosphate buffer, pH 7.3; capillaries, 47 cm; 5 s low-pressure sample injection; 25 °C; separation voltage, 15 kV. Absorbance detection at 280 nm for (a–c), 214 nm for (d).

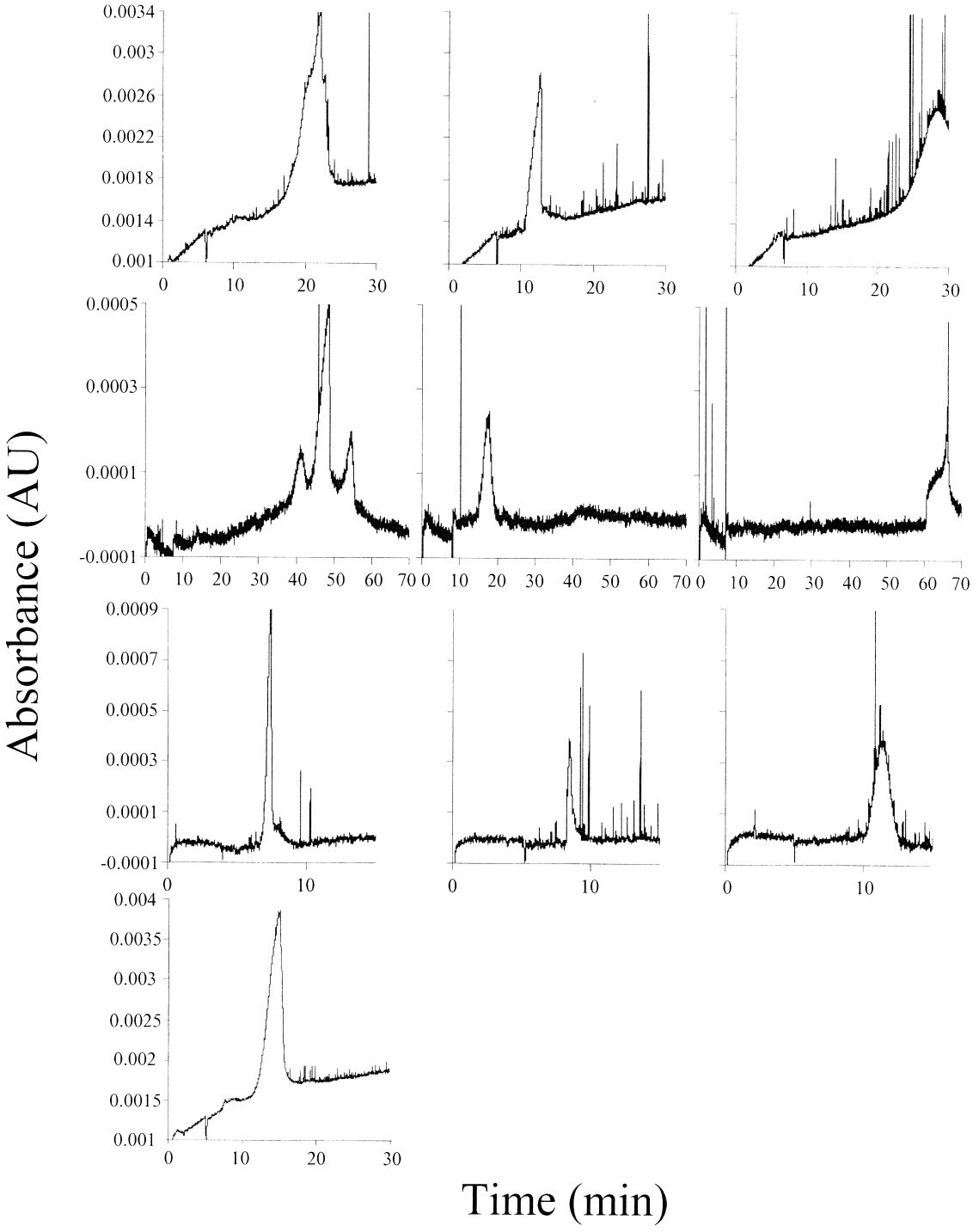

Fig. 3. Results for individual casein proteins. Left to right: αCN, βCN, κCN. Top to bottom: a **1**-coated capillary (75 μm I.D., 100 μ*M* protein, EOF=6.5 min), a **2**-coated capillary (75 μm I.D., 63 μ*M* protein, EOF=7.3 min), a bare capillary (50 μm I.D., 100 μ*M* protein, EOF=3.6 min), and a **3**-coated capillary (shown for αCN only, 75 μm I.D., 63 μ*M* protein, EOF=6.2 min). Mobile phase: 10 m*M* phosphate buffer, pH 7.3; capillaries, 47 cm; 5 s low-pressure sample injection; 25 °C; separation voltage 15 kV.

3.2. Separations of milk samples

A 25% solution of skim milk run on a **2**-coated capillary gave an electrochromatogram (Fig. 4a) that is very similar to that obtained for the mixture of purified milk proteins using the same type of capillary (Fig. 2a). The peaks were identified by co-injecting each of the individual proteins with the milk sample. The elution order of the baseline-resolved proteins in the milk sample was the same as that for the mixture of purified proteins. Only two peaks were clearly resolved for αCN, possibly because the purified protein and the milk sample came from different bovine breeds that may differ in their contents of genetic variants.

For the separation of the mixture of purified proteins (Fig. 2a), κCN gave a broad peak that was not completely separated from αCN. For the milk sample (Fig. 4a), the κCN peak is much sharper and completely resolved from αCN. This may be due to differences between the commercially purified κCN and the κCN found in the skim milk sample, or to the presence of other components in the milk sample that change the aggregation properties of κCN.

The milk sample was also run on a **3**-coated capillary (Fig. 4b). The peaks for βCN, βLgA, and βLgB could not be resolved, and αCN eluted as a single, broad peak. κCN was completely resolved from αCN, but interestingly, it eluted much later than on the **2**-coated capillary. This suggests that there may be interactions between κCN as it exists in milk and the scrambled sequence oligonucleotide that did not occur for the purified protein.

The electropherogram of the milk sample on a bare capillary (Fig. 4c) was generally similar to the electrochromatogram obtained using the **3**-coated capillary. The βCN, βLgA, and βLgB peaks could not be resolved, and αCN eluted as a single, broad peak. κCN was still completely resolved from αCN, but the elution time was much shorter for the bare capillary than for either of the oligonucleotide-coated capillaries. As for the individual proteins, the elution order of the proteins in the milk sample varied over the lifetime of the capillary.

Reproducibility of the electrochromatograms of the milk sample run on a **2**-coated capillary is shown in Fig. 5. αLa, βCN, βLgB, and βLgA were baseline resolved each time, and their retention times did not vary significantly. However, the peak areas changed from run to run, most likely due to inconsistent suspension of the untreated milk sample. This indicates the difficulty in quantitation using this method, and suggests a need for sample pre-treatment if quantitation of each protein is desired. αCN consistently gave two resolved peaks, but the peak shapes changed slightly. κCN gave the most inconsistent result; the retention time was later and the band-broadening more severe for each successive run, indicating that κCN may adsorb to any bare, exposed silica surfaces or may interact very strongly with the oligonucleotide. These results suggest that for the complex, unstable caseins, which are known to associate and form micelles due to their high hydrophobicities and unusual charge distributions [43], highly reproducible separations are difficult using this technique. Treating the milk samples to disrupt the micelles and to create more uniform suspensions may improve the reproducibility to the point where this technique could be valuable for the separation and detection of milk proteins in the agricultural industry.

3.3. Heat-denatured milk experiments

In order to determine if CEC using the **2**-coated capillary could be used to detect thermal damage, the milk sample was run after being incubated at 90 °C for 25 min and cooled to room temperature. The electrochromatogram (Fig. 6) was very different from those obtained for an unheated sample. The same treatment was performed on each of the individual milk proteins in an attempt to identify the peaks (Fig. 7). Based on these results, tentative assignments were made for the peaks in Fig. 6 and are given in the figure caption.

When run individually, the heated sample of βLgA (Fig. 7b) eluted as a small peak at approximately 5 min, and the samples of αLa (Fig. 7a), βLgB (Fig. 7c), and βCN (Fig. 7d) each eluted as broad peaks at approximately 9 min. These proteins most likely correspond to the first very small, broad peaks/shoulders in the electrochromatogram of the heat-denatured milk sample (Fig. 6). Denatured αCN (Fig. 7e) eluted as a broad peak at approximately 15 min and a sharp peak at approximately 25 min. These most likely correspond to the broad peak at

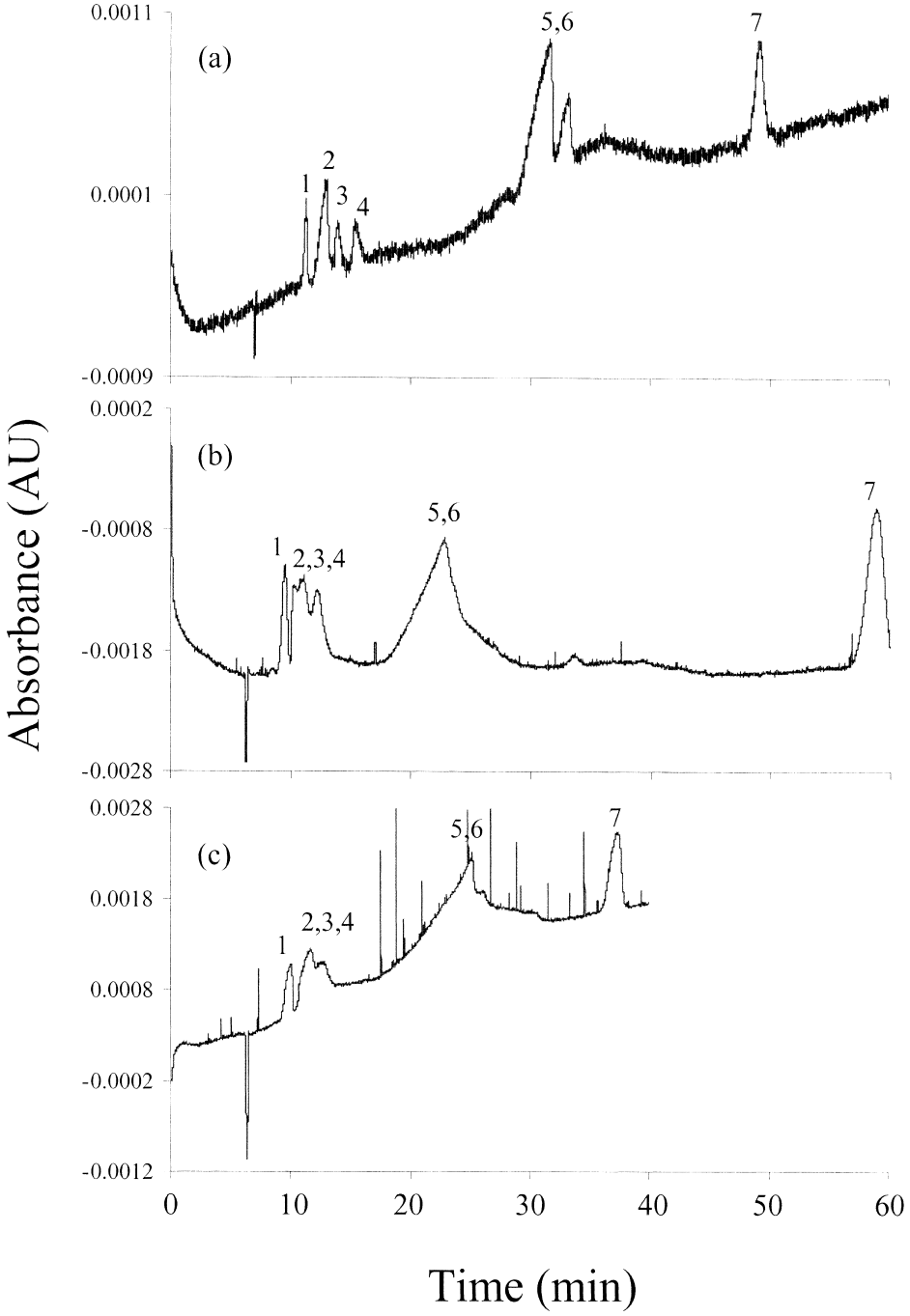

Fig. 4. Results for bovine milk obtained using (a) a **2**-coated capillary (EOF=7.0 min); (b) a **3**-coated capillary (EOF=6.3 min); and (c) a bare capillary (EOF=6.4 min). Sample: untreated bovine skim milk, 25%, in 10 m*M* phosphate buffer. Peaks: 1, αLa; 2, βCN; 3, βLgB; 4, βLgA; 5,6, αCN; 7, κCN. Mobile phase: 10 m*M* phosphate buffer, pH 7.3; capillaries, 47 cm, 75 μm I.D.; 5-s low-pressure sample injection; 25 °C; separation voltage 15 kV. Negative peaks at 7.0, 6.3, and 6.4 min in (a), (b), and (c), respectively, are due to a change in refractive index when the sample plug migrates past the detector, and serve as EOF markers.

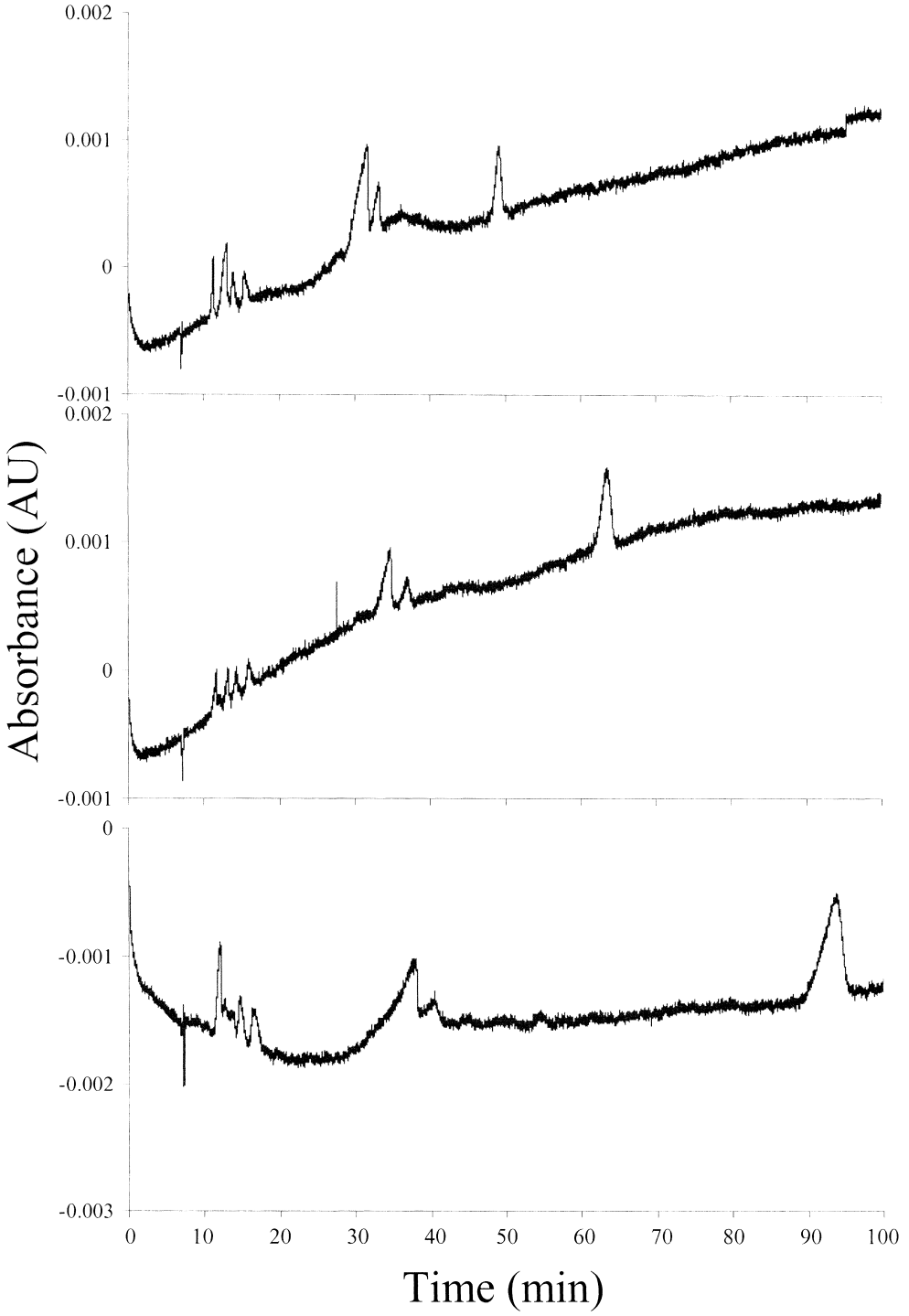

Fig. 5. Reproducibility of electrochromatograms of bovine skim milk, 25%, run on a **2**-coated capillary under the same conditions as in Fig. 4.

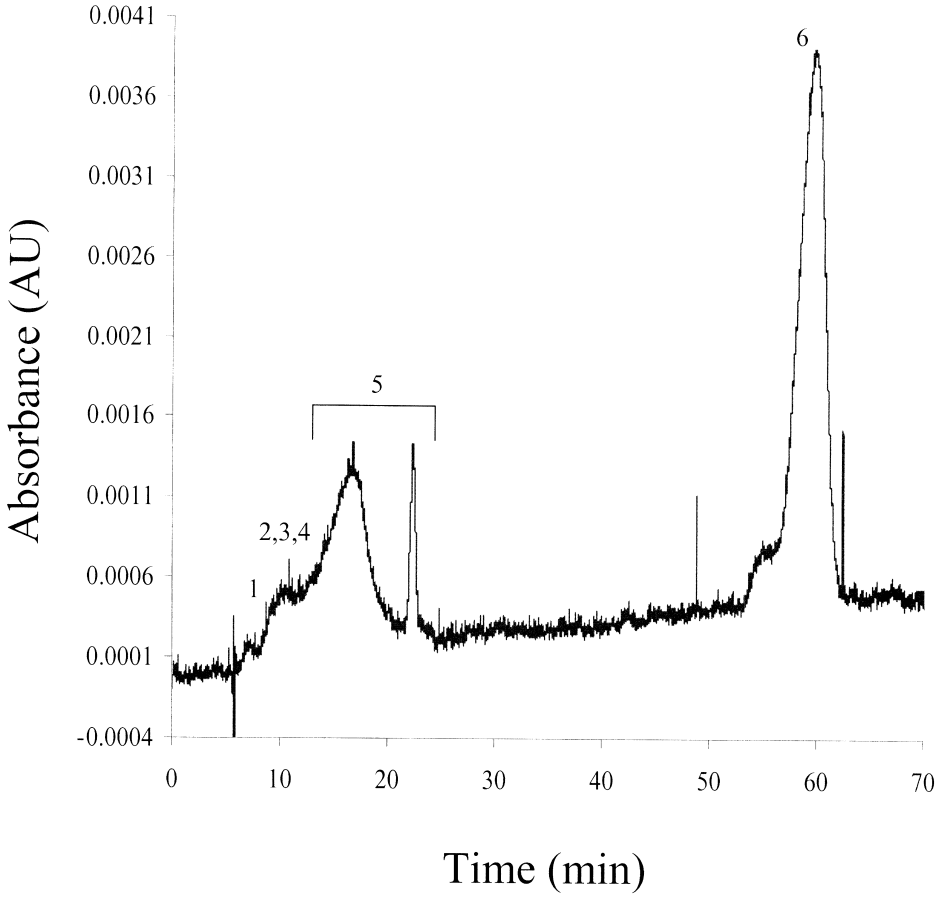

Fig. 6. Electrochromatogram of heat-denatured bovine milk run on a **2**-coated capillary. Sample: bovine skim milk, 25%, in 10 m*M* phosphate buffer, heated at 90 °C for 25 min, then cooled to room temperature. Peak identities based on results for individual milk proteins (see Fig. 7, below): 1, βLgA; 2, αLa; 3, βCN; 4, βLgB; 5, αCN; 6, unknown aggregate. Conditions same as in Fig. 4.

15–18 min and the sharp peak at 23 min in the electrochromatogram of the heated milk sample (Fig. 6). The electrochromatogram of the heated sample of κCN (Fig. 7f) indicated that heated κCN strongly adsorbs to the stationary phase and exposed capillary surface upon denaturation, and no distinctive peaks that correspond to those in the electrochromatogram of the heated milk sample were observed. This again may be due to differences between the commercially purified κCN and the κCN found in the skim milk, but is more likely due to the different properties of κCN in the presence of the other proteins in the milk sample. κCN is known to interact with other caseins through hydrophobic interactions, hydrogen-bonding, and electrostatic interactions, and it has been identified as the factor responsible for micelle stabilization [43].

None of the individual proteins corresponded to the third peak in Fig. 7; however, it has been shown that severe heating may cause irreversible association of unfolded whey proteins with other whey proteins or with casein micelles [44]. It is likely that the peaks in the electrochromatogram of the heat-denatured milk, particularly the third peak, correspond to various aggregates of denatured proteins with each other and with other milk components.

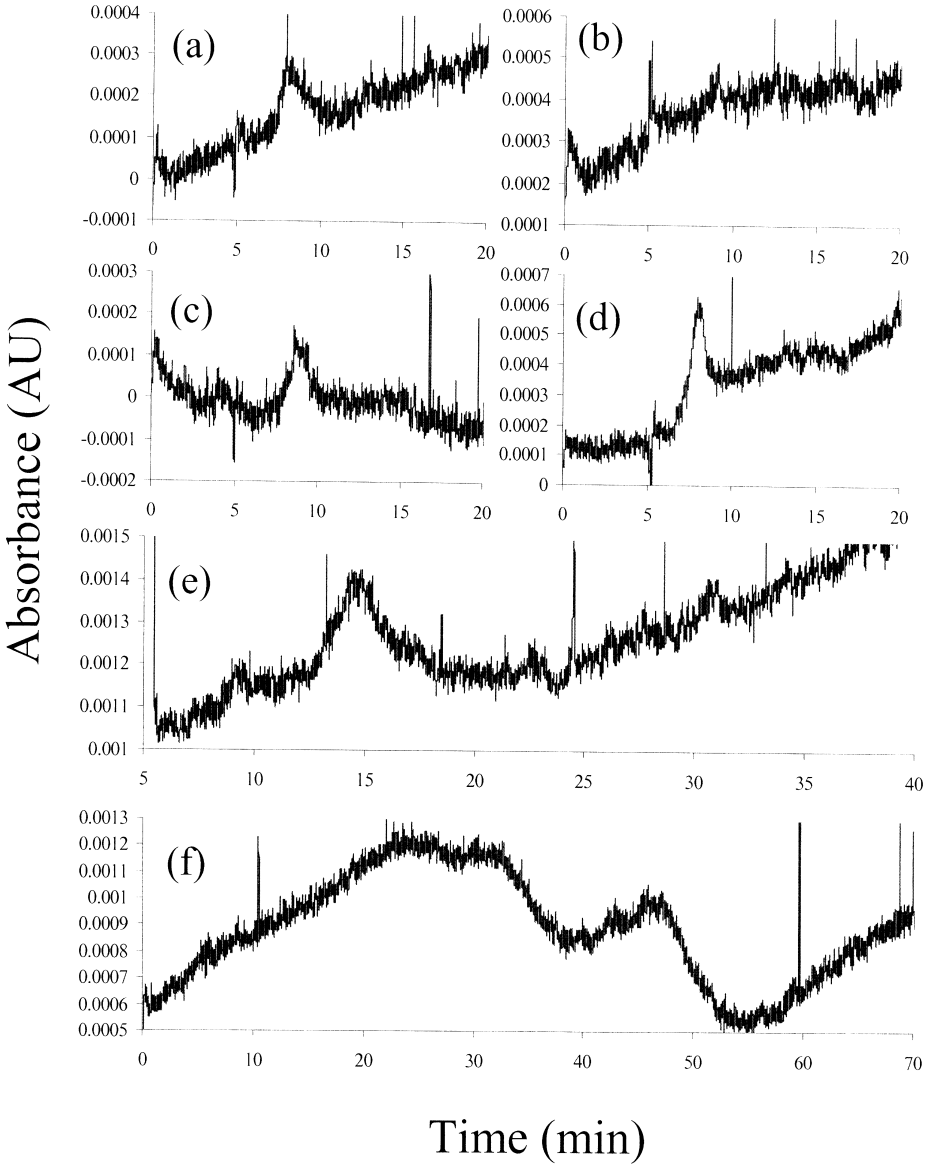

Fig. 7. Electrochromatograms of individual, heat-denatured bovine milk proteins run on a **2**-coated capillary, treated as described in Fig. 6 and run under the same conditions: (a) 20 μ*M* αLa, (b) 25 μ*M* βLgA, (c) 25 μ*M* βLgB, (d) 60 μ*M* βCN, (e) 60 μ*M* αCN, and (f) 60 μ*M* κCN.

4. Conclusions

OTCEC using G-quartet stationary phases resolved eight major proteins found in bovine milk. The separations were performed without the traditional, harsh sample pre-treatments that are used to isolate the whey proteins from the caseins or to disrupt the casein micelles. The G-quartet structure

of the oligonucleotides appears to play a role in the separation since a capillary coated with a non-G-quartet-forming oligonucleotide provided much less resolution. Furthermore, three peaks were resolved for the individual α-casein using the 4-plane G-quartet. Some indication of the three peaks was also observed with the 2-plane G-quartet, but not with the non-G-quartet-forming oligonucleotide or the bare capillary. This suggests that the G-quartet stationary phases are sufficiently sensitive to structural and conformational differences to allow separation of very similar protein structures, such as those arising from genetic variation or post-translational modification. Under the mild conditions of the separations, it was possible to detect thermal denaturation of the proteins using the 4-plane G-quartet stationary phase.

Acknowledgements

This material is based upon work supported by the Cooperative State Research, Education, and Extension Service, US Department of Agriculture, under Agreement No. 2001-35503-10731.

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