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Separation of isoforms of *Serratia marcescens* nuclease by capillary electrophoresis

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ABSTRACT

Three *S. marcescens* nuclease isoforms differing mainly in charge (native nuclease with p*I* 6.8 and two minor isoforms with p*I* 7.3 and 7.4) were separated using several different modes of high-performance capillary electrophoresis. Separation of the isoforms by free solution capillary electrophoresis was unsatisfactory. Separation by micellar electrokinetic capillary chromatography was therefore investigated in detail and the method optimized with respect to pH and sodium dodecyl sulphate concentration; in addition, the effect of adding various substances to control dispersion and avoid analyte adsorption at the capillary wall was examined. Under optimal conditions there was almost complete baseline separation of the two isoforms with basic p*I* whereas there was only partial separation of the native form and the isoform with p*I* 7.4. With capillary isoelectric focusing there was complete baseline separation of the native nuclease and the other two isoforms.

INTRODUCTION

The use of high-performance capillary electrophoresis (HPCE) for protein analysis in biotechnological production is gaining increasing interest. Since HPCE is rapid and separates molecules by virtue of differences in their charge and hydrodynamic properties, it provides an advantageous alternative to existing analytical techniques such as gel electrophoresis, which is rather time consuming, and reversed-phase HPLC, which separates according to solute hydrophobicity and is therefore often unsuitable for separating globular proteins. Although interest in HPCE particularly concerns *in process* analysis, the possibilities for applying HPCE to biotechnological processes are largely unexplored.

Serratia marcescens nuclease is an active globular molecule with a p*I* of 6.8 [1], and a molecular mass of 26 705.8 as calculated from the amino acid sequence [2]. The enzyme is stable within the pH range 6–8 for at least 1/2 h at temperatures from 2 to 37°C [3], but is thermally degradable above 40°C [4]. Two closely related isoforms of the enzyme having p*I* 7.3 and 7.4 have recently been isolated from *S.*

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marcescens fermentation broth; both are N-terminal split variants of the native enzyme encoded from the nucleotide sequence of the *nuc* gene. The p*I* 7.4 isoform lacks Asp–Thr–Leu while the p*I* 7.3 isoform only lacks Asp [1].

Hitherto isoelectric focusing has been used to detect these isoforms in the fermentation broth and during isolation and purification of the enzyme. In the present study we evaluated the efficacy of the following modes of HPCE in separating these closely related and enzymatically active nuclease isoforms: free solution capillary electrophoresis (FSCE), micellar electrokinetic capillary chromatography (MECC) using the surfactant sodium dodecyl sulphate (SDS), and capillary isoelectric focusing (cIEF) using ampholytes. MECC has previously been used mainly for analyzing small organic molecules, especially small neutral organic molecules that are difficult to separate by FSCE [5,6]. In this study the separation of macromolecules such as nuclease isoforms by MECC was optimized with respect to pH, buffer type, SDS concentration, and by manipulation of the micellar buffer by the addition of methanol, hydroxypropylmethylcellulose, and mono- and divalent ions. A recent modification of MECC in which microemulsions are used as the medium [7,8] was also examined. The results obtained using MECC were compared to those obtained using FSCE and cIEF.

EXPERIMENTAL

Preparation of nuclease

Nuclease was produced and purified from two *S. marcescens* B10M1 fermentations as previously described [9,10]. The isoforms were partially separated by DEAE-cellulose chromatography, elution being undertaken using a narrow pH gradient (pH 8.25–7.2). The first peak to elute contained in one case a major basic isoform with p*I* 7.3, and a minor basic isoform with p*I* 7.4, and in the other case only the isoform with p*I* 7.4. The main peak, which eluted last, contained the native nuclease with p*I* 6.8. Fractions collected from the two peaks from each of the fermentations were dialysed against water and lyophilized prior to HPCE.

Preparation of reagents

All reagents were of analytical grade. The pH of the Tris buffer was adjusted using H_2SO_4 and that of citrate buffer using citric acid. SDS solutions were prepared by adding SDS (Pharmacia Biotechnology, Allerød, Denmark) to the buffers, usually at a concentration of 25 m*M*. Hydroxypropylmethylcellulose (HMC) (Merck, Darmstadt, Germany) was solubilized in 40 m*M* Tris buffer, pH 7.5, while stirring overnight and then filtered through a 45-μm membrane filter (Millipore, Guyancourt, France). HMC buffers containing 25 m*M* SDS were prepared with HMC concentrations from 0.05 to 0.25% (w/v).

Three microemulsions were investigated, their composition being as described by Watarai [8]: (A) water–SDS–1-butanol–heptane (89.3:3.3:6.6:0.8, v/w/w/w), (B) water–SDS–1-butanol–heptane (76.0:6.3:12.7:5.0, v/w/w/w); (C) water–SDS–1-butanol–heptane (65.1:9.3:18.6:7.0, v/w/w/w) (the water was 40 m*M* Tris–H_2SO_4, pH 7.5). These microemulsions were chosen according to a ternary phase diagram [11] of the system water–SDS–1-butanol–heptane such that each gave a single-phase microemulsion of oil in water. In addition, the composition of microemulsion A was modified with respect to SDS and water, that of A_1 being water–SDS (91.9:0.7) and that of A_2 being water–SDS (91.1:1.5).

The reagents used for cIEF were prepared according to the method described by the HPCE equipment manufacturer [12]. The ampholyte used was Servalyt 3–10 (Serva, Heidelberg, Germany).

High-performance capillary electrophoresis (HPCE)

HPCE was performed using an Applied Biosystems Model 270 A system (Applied Biosystems, Foster City, CA, USA) comprising an untreated capillary tube (52 cm × 50 μm I.D. × 300 μm O.D.), a high-voltage power supply (0–30 kV), an autosampler with hydrodynamic in-

jection, and a variable-wavelength UV–Vis absorbance detector located on the capillary tube, the whole system being coupled to an LKB 2220 integrator (LKB, Bromma, Sweden).

The polarity of the internal power supply was set such that the components of the sample would migrate towards the cathode, and hence the detector. At the start of each analysis the electrode reservoirs at the detector and inlet ends of the capillary were filled with running buffer and the capillary then flushed (by vacuum displacement at 67.6 kPa) with 0.1 *M* NaOH for 2 min followed by 0.1 *M* HCl for 2 min. Running buffer was then introduced for 3 min to replace the flush fluid and a small sample zone introduced for 1.0 s with 16.9 kPa vacuum. The two ends of the capillary were then immersed in the buffer reservoirs and a high voltage (typically 3–20 kV) applied along the capillary. Electropherograms were made by measuring absorbance at 214 nm, using a range of 0.02 absorbance units full scale and a rise time of 1.0 s.

Prior to HPCE the nuclease samples were solubilized in a solution of electrophoresis buffer and water (1:3, v/v) so as to obtain stacking conditions during electrophoresis. The total protein concentration in the samples was approximately 0.5 μg/μl buffer.

It was attempted to separate the three nuclease isoforms by means of the following modes of HPCE: FSCE, MECC and cIEF.

Separation by free solution capillary electrophoresis

This is the most simple and common mode of HPCE, separation being based solely on differences in the electrophoretic mobility of the analytes. Since pH of the running buffer plays a crucial role in determining the selectivity of separation, different types of buffers [Tris, tricine, phosphate, citrate and 2-(N-cyclohexylamino)ethansulfonic acid (CHES)] with different pH and molarity were tested with respect to separation of the three nuclease isoforms. The electric field strength and the ionic strength of the buffer were chosen such that Joule heating would not cause thermal degradation of the nuclease [4].

Separation by micellar electrokinetic capillary chromatography

As separation by FSCE was unsatisfactory (see Results), an extensive investigation was undertaken of separation by MECC, a more sophisticated mode in which analyte separation is based on a combination of differences in electrophoretic mobility and degree of partitioning with negatively charged SDS micelles. The method was optimized with respect to pH, buffer type, SDS concentration, micelle composition, etc.

Capillary isoelectric focusing

Separation was carried out using the method developed by the HPCE equipment manufacturer [12]. Since effective focusing is prevented by the electroosmotic flow that characteristicly takes place when using uncoated fused-silica capillaries, focusing was performed using a coated capillary [72 cm (length to the detector 50 cm) × 50 μm I.D. × 375 μm O.D.]. After flushing as described earlier, the capillary was loaded (8.3 min) with an electrolyte solution comprised of 20 m*M* NaOH containing 0.4% methylcellulose (Sigma, St. Louis, MO, USA) that had been filtered through a 0.2-μm membrane filter and centrifuged prior to use. The capillary was then loaded with the same buffer containing 0.5% Servalyt 3–10 (Serva) to a distance about 40 cm from the inlet end. The wavelength was adjusted to 280 nm since ampholytes strongly absorb UV light between 200 and 250 nm. The sample was injected using 16.9 kPa vacuum for 17.2 s and then more ampholyte solution injected for 6 s such that the front of the first ampholyte solution was near the detector window. Focusing was undertaken using 100 m*M* H_3PO_4 containing 0.4% methylcellulose as the anode electrolyte and applying a voltage of 30 kV until the current had stabilized at 0–1 μA (5.8 min).

Detection/mobilization was performed by turning on the 16.9 kPa vacuum and high voltage (30 kV) for 28.8 min with the anode end of the capillary still immersed in the anode solution (100 m*M* H_3PO_4) and monitoring the detector signal (indicating protein peaks) with the integrator. The relative mobility of the separated components is linearly related to their p*I* [12].

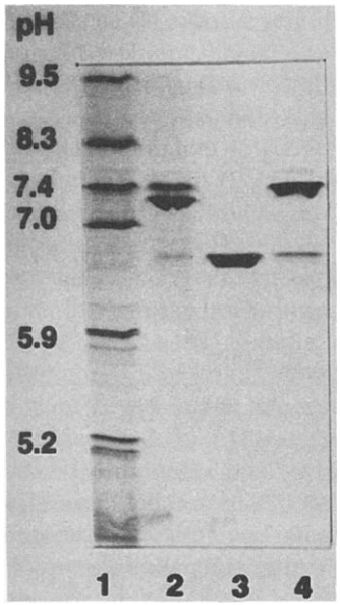

Fig. 1. Conventional isoelectric focusing of nuclease isoforms. Nuclease isolated from *S. marcescens* B10M1 was purified on a phosphocellulose column and the native nuclease, having p*I* 6.8, separated from the more basic isoforms by DEAE-cellulose chromatography. Lanes: 1 = p*I* marker; 2 = sample in which the p*I* 7.3 isoform is the major component and the p*I* 7.4 isoform the minor component; 3 = native nuclease with p*I* 6.8; 4 = sample in which the isoform p*I* 7.4 is the major component.

Conventional isoelectric focusing (IEF)

IEF was performed horizontally in 0.3 mm thick 1% IEF agarose (Pharmacia) on a LKB Multiphor 2117 (Pharmacia LKB Biotechnology) according to Biedermann and Nielsen [13]. Samples of the two peaks eluted by DEAE-cellulose chromatography were applied to the gel in a concentration of 1 μg/μl (10 μl) and the focused proteins stained with Coomassie Brilliant Blue G-250.

RESULTS AND DISCUSSION

Conventional isoelectric focusing in an agarose gel (Fig. 1) revealed that one sample of the purified nuclease contained the native nuclease with p*I* 6.8 (lane 2) and that two contained the isoforms with p*I* 7.3 and 7.4, albeit in different amounts (lane 1 and 3). These three samples were analyzed by HPCE, both separately and as a mixture of the samples shown in lanes 2 and 3. The difference in the primary structure of the isoforms is illustrated in Fig. 2.

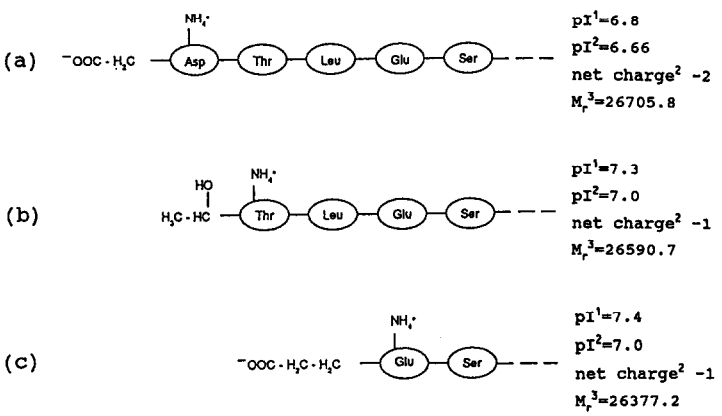

Fig. 2. Physico-chemical properties of the N-terminal amino acid residues of the three nuclease isoforms. (a) Native nuclease with p*I* 6.8 containing 245 amino acids residues; (b) isoform with p*I* 7.3 lacking the negatively charged amino acid aspartic acid; (c) isoform with p*I* 7.4 lacking both the aspartic acid and the two uncharged amino acids residues, threonine and leucine. [1]Isoelectric point determined by analytical IEF; [2]isoelectric point calculated from the amino acid sequence using the GCG-program (University of Wisconsin, USA); [3]molecular mass calculated from the amino acid sequence average value based on the natural abundance of isotopes [2].

Separation by FSCE

The separation efficiency obtained with Tris buffer at pH 7.5, 8.0 and 8.5 is shown in the electropherogram (Fig. 3). The best resolution was obtained at pH 8.0; however, isoform separation was poor, the sample resolving into only two partially separated peaks with the basic isoforms (p*I* 7.3 and 7.4) eluting together in the first peak. The elution order indicated on the figure was determined by analyzing each of the isoforms separately. Thus separation of the nuclease isoforms, all of which have a p*I* close to neutrality, was unsatisfactory with this mode of HPCE.

Separation by MECC

The effect of pH on isoform separation in 40 m*M* Tris buffer containing 25 m*M* SDS is illustrated in Fig. 4a–c. Separation was better than that obtained with FSCE, particularly at pH 7.1, which is between the p*I* of the isoforms, and at pH 7.5, which is just above the p*I* of the most basic of the isoforms. Similar separation was obtained using a citrate buffer at pH 7.1 instead of Tris–H_2SO_4 (Fig. 4d), and when using a tricine buffer.

With MECC the order of migration is the reverse of that seen with FSCE (Fig. 5). Because of electrostatic repulsion from the more slowly migrating micelle phase, native nuclease with p*I*

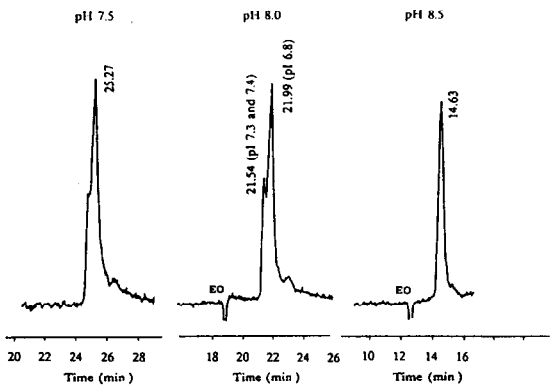

Fig. 3. Separation of nuclease isoforms by free solution capillary electrophoresis at different pH values. The electrophoresis buffer used was 40 m*M* Tris–H_2SO_4. The sample containing the three isoforms was introduced by applying a 16.9 kPa vacuum at the detector end for 1.0 s. A voltage of 5 kV was applied during electrophoresis. EO = electroosmotic flow.

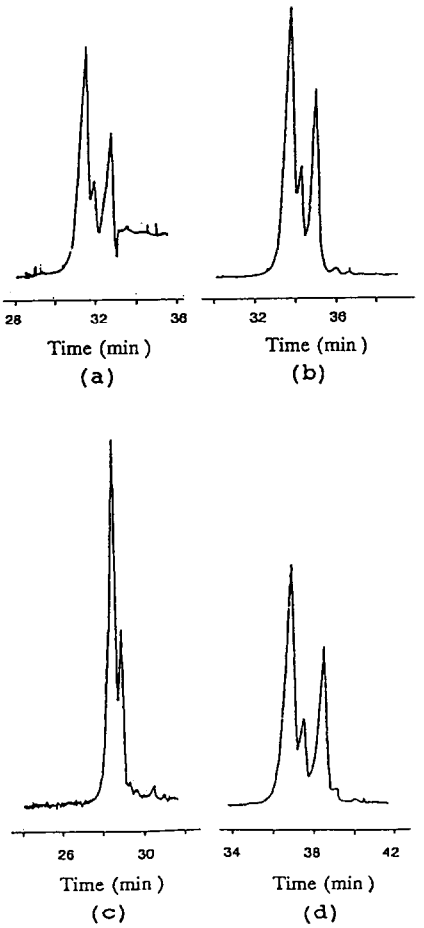

Fig. 4. Separation of nuclease isoforms by micellar electrokinetic capillary chromatography at different pH. The sample containing the three isoforms was introduced by applying a 16.9 kPa vacuum for 1.0 s at the detector end. 10 kV was applied during electrophoresis. (a–c) 40 m*M* Tris–H_2SO_4 electrophoresis buffer containing 25 m*M* SDS. (a) pH 7.1; (b) pH 7.5; (c) pH 8.5; (d) 40 m*M* citrate buffer, pH 7.1.

6.8, which has the most negative net charge, probably spends more time in the bulk phase than the more basic isoforms, thus explaining why it elutes first. The negatively charged side chain of glutamic acid at the N-terminal of the isoform with p*I* 7.4 (Fig. 2) may explain why it migrates faster than the isoform with p*I* 7.3; since the latter has uncharged Thr at the N-terminal, it probably has a greater affinity for the micelles and hence a slower migration velocity. Conditions other than electrostatic repulsion and attraction may also affect the elution order,

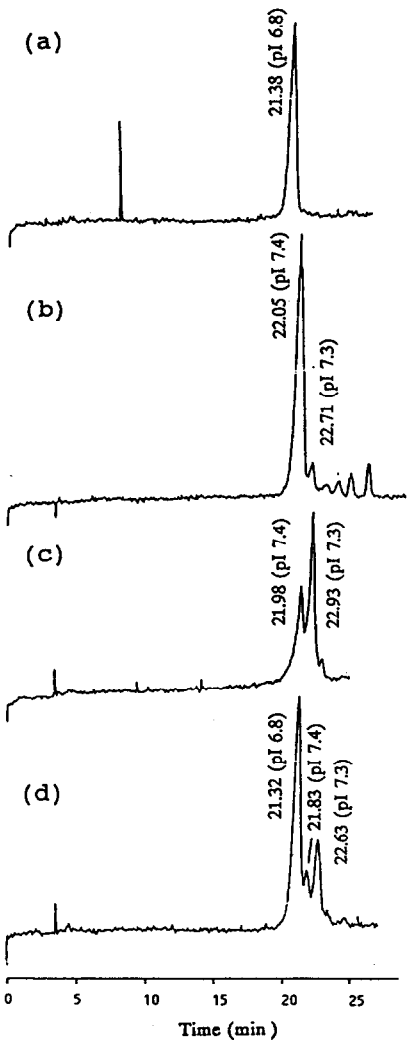

Fig. 5. Evaluation of the migration order of nuclease isoforms during separation by micellar electrokinetic capillary chromatography. The electrophoresis buffer was 40 m*M* Tris–H_2SO_4, pH 7.5, containing 25 m*M* SDS. Samples were introduced by applying a 16.9 kPa vacuum for 1.0 s at the detector end. 15 kV was applied during electrophoresis. (a) Native nuclease, p*I* 6.8; (b) sample in which the 7.4 isoform is the major component and the 7.3 isoform the minor component; (c) sample in which the 7.3 isoform is the major component and the 7.4 isoform the minor component; (d) mixture of (a) and (c).

however, *e.g.* hydrophobic interaction and electrophoretic migration of the analytes [14].

The influence of micelle concentration on separation was studied by using from 15 to 75 m*M* SDS in 40 m*M* Tris buffer, pH 7.5. The

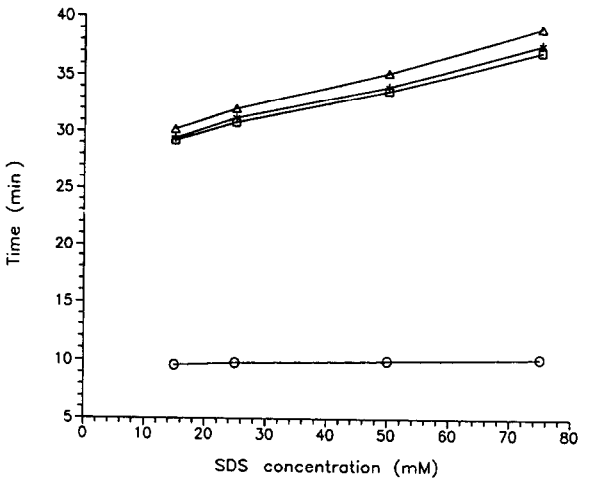

Fig. 6. Influence of SDS concentration on electrophoretic migration time during micellar electrokinetic capillary chromatography with 40 m*M* Tris–H_2SO_4 buffer, pH 7.5, containing SDS. Voltage applied was 10 kV. ○ = Neutral components; □ = native nuclease with p*I* 6.8; * = isoform with p*I* 7.4; △ = isoform with p*I* 7.3.

optimal SDS concentration was between 25 and 50 m*M*, this being in agreement with the findings of Sepanik and Cole [5]. Fig. 6 shows that with increasing SDS concentration electroosmotic flow remains unchanged but there is a linear increase in electrophoretic migration time, a finding in concert with that of Terabe *et al.* [14]. The constancy of electroosmotic flow suggests that SDS has no effect on the zeta potential of the capillary/micelle phase. At the highest SDS concentration studied, 75 m*M*, the peaks began to broaden, possibly because of dispersion due to thermal effects.

Modification of the aqueous mobile phase by the addition of methanol has been reported to enhance MECC separation efficiency for small molecules such as aliphatic amines and aflatoxins [15]. Adding organic solvents both extends the elution range by reducing the electroosmotic flow velocity, and increases the solubility of hydrophobic compounds in the mobile phase, thereby reducing partititioning into the micelles. In the present study the addition of methanol in concentrations from 0 to 25% (v/v) actually reduced the electroosmotic flow and there was a small decrease in migration velocity relative to the electroosmotic flow. However, since the

extension of the elution range was inadequate to compensate for the reduction in isoform partitioning to the micelles, separation efficiency did not improve. That the degree of separation was lower and the peaks broader at higher methanol concentrations is probably attributable to methanol-induced polydispersion in the micelles [16].

Addition of hydroxypropylmethylcellulose to the SDS buffer was expected to increase the viscosity of the mobile phase and thereby lower the electroosmotic flow velocity; however, HMC did not enhance resolution of the isoforms at the concentrations used (0.05 to 0.25%). The expected enhancement should be due to reduced dispersion [17].

In order to reduce adsorption of the isoforms to negatively charged silanol groups on the capillary wall, NaCl was added to the buffer solution at concentrations from 10 to 100 m*M*; the theory was that Na^+ ions would compete with the analytes for the adsorption sites. However, although slightly better separation of the two basic isoforms was obtained when using 20 m*M* NaCl, separation of the isoforms with p*I* 6.8 and 7.4 was reduced; at higher NaCl concentrations the peaks broadened and migration velocity slowed.

The presence of divalent metals may enhance separation because divalent metals electrostatically attracted to the surface of negatively charged micelles could interact with the components to be separated [18,19]. However, although nuclease requires Mg^{2+} ions for its activity and hence might be expected to interact with metal sites on micelles, the addition of Mg^{2+} ions did not influence isoform separation and in fact the peaks actually broadened. The latter may be due to the additional adsorption of metal ions and consequently nuclease to the metal–fused silica surface.

One problem in applying MECC to large proteins is the rather small size of the SDS micelles (diameter 40 Å [5]) —only part of the protein molecule can interact with the hydrophobic tails of the SDS molecules. We therefore attempted to utilize an oil in water microemulsion which permits the formation of larger micelles [7,8,20]; this was comprised of Tris–H_2SO_4 buffer, pH 7.5, 1-butanol, SDS and heptane. In such a pseudophase system the heptane oil droplet will be surrounded by both SDS molecules and butanol, thereby enhancing micellar diameter. Alcohol may loosen the micelle structure and thereby improve its mass transfer kinetics as compared with the more rigid micelles formed by SDS alone. The effect of varying microemulsion composition on the separation of the three nuclease isoforms was examined using 3 water–butanol–SDS–heptane systems, A, B and C, as described above (see Experimental). System A, which was characterized by a high percentage of water and a low percentage of the other components, provided the best separation although even then the three isoforms were found in one broad peak with a split in the top. Increasing the composition of SDS and reducing that of water only slightly improved in separation efficiency. It can thus be concluded that this microemulsion is unsuitable for the separation of large proteins such as nuclease isoforms.

Separation by cIEF

Separation of the three isoforms by cIEF, another means of achieving charge-based separation in HPCE, was also investigated. After the isoforms had been focused to a particular position according to their isoelectric points, the entire contents of the capillary were mobilized past the detector so as to yield an electropherogram in which time and p*I* are related. The anode electrolyte was acidic and the cathode electrolyte basic. In the cIEF electropherogram shown in Fig. 7, it can be seen that there is complete baseline separation of native nuclease and the two basic isoforms with p*I* 7.3 and 7.4, separation therefore being much more efficient than with FSCE and MECC. The elution order is that expected with cIEF, *i.e.* the basic isoforms eluted first. Full separation of the two basic isoforms was not achieved however, the difference in p*I* and primary structure being too small. Their separation probably requires further optimization of the conditions and the use of a narrower pH gradient. The separation of nuclease isoforms achieved with cIEF is virtually the same as that achieved with IEF in agarose gels (Fig. 1); however, cIEF is much easier and

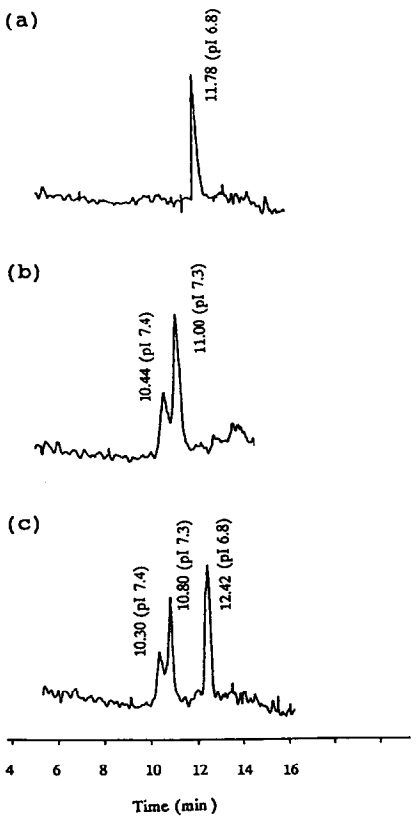

Fig. 7. Capillary isoelectric focusing of nuclease isoforms. The carrier ampholyte was Servalyte 3–10. (a) Native nuclease with p*I* 6.8; (b) sample in which the p*I* 7.3 isoform is the major component and the p*I* 7.4 isoform the minor component; (c) a mixture of (a) and (b) (1:1).

faster to perform, gel casting and lengthy staining procedures being obviated, and ampholyte consumption considerably reduced.

CONCLUSIONS

HPCE proved to be a promising new method for analyzing closely related proteins. However, optimization of separation is difficult compared to conventional electrophoresis because of the numerous factors involved. Separation of the nuclease isoforms by MECC was more efficient than with FSCE, the best results being obtained with a 40 m*M* Tris buffer, pH 7.5, containing 25 m*M* SDS but lacking any viscosity-increasing or complexing agents. Best separation was obtained using cIEF, which enabled complete baseline separation of the native nuclease with p*I* 6.8 and the isoform with p*I* 7.3, and nearly complete baseline separation of the p*I* 7.3 and 7.4 isoforms.

The main potential use of HPCE in biotechnology could be for the on-line monitoring of product formation and product quality during fermentation and downstream processing. Another potential use is for the separation of proteins under conditions that preclude using other techniques, in order to enable their further analysis. In this case fractionated proteins would have to be collected and if SDS and ampholytes have been used for the separation, they would have to be removed.

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