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Analysis of the quaternary structure of catalase by capillary zone electrophoresis

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Abstract

Analysis of the subunit composition of catalase was achieved by capillary zone electrophoresis. Denaturation of catalase with 34.7 m*M* sodium dodecyl sulfate (SDS) before electrophoretic analysis reveals that the protein is composed by two different classes of subunits. These results were confirmed by polyacrylamide gel electrophoresis. Micellar electrokinetic capillary chromatography also confirmed the occurrence of two classes of subunits when native catalase was injected into the capillary, although a significant decrease in efficiency was observed. However, very low efficiency and resolution were obtained when SDS was included in the running buffer as a modifier.

1. Introduction

High-performance capillary electrophoresis (HPCE) is a relatively new tool for the determination of biologically active molecules. In addition to the capability of attaining separation efficiencies higher than those obtained with high-performance liquid chromatography (HPLC) or isolectrofocusing in a pH gradient (IPG), it has the potential to overcome several of the problems associated with slab gel electrophoresis systems [1]. Molecules such as amino acids, small peptides, proteins and oligonucleotides have been separated under conditions that provide numbers of theoretical plates in the hundreds of thousands and more.

Numerous efforts have been made to separate proteins by free solution capillary electrophoresis (FSCE) as they show potential adsorption to the capillary walls. Such adsorption leads to considerable peak broadening and asymmetry, making it difficult to attain the efficiencies predicted by theory. Optimized separation methods include coating the capillary surface [2,3], addition of modifiers [4,5] and changes in the pH and composition of the electrolyte [6,7].

In a previous paper [7], we demonstrated the importance of the temperature, voltage and ionic strength of the electrolyte buffer in the separation of acidic proteins from a solution mixture by FSCE. Depending on the separation problem, it may be desirable to work at low pH (below the isoelectric point of the proteins) in a cationic regime or, conversely, it may at times be advantageous to work at pH values above the isoelectric point of the proteins, rendering them negative and migrating against the electroosmotic flow (EOF).

A question to be resolved consists in the application of CZE to separate different protomers of a polymeric protein, such as catalase [8], in a way similar to conventional sodium dodecyl

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sulfate polyacrylamide gel electrophoretic (SDS-PAGE) separations. Catalase has an M_r of 250 000 and a p*I* of 5.4 [8] and it is composed of four protomers. In this work, we tried to analyse the protomer separation of catalase by CZE and PAGE by using SDS as a modifier of the running buffer or, alternatively, as a dissociative agent.

2. Experimental

2.1. Apparatus

A Hewlett-Packard (Waldbronn, Germany) three-dimensional capillary electrophoresis system was used. Fused-silica capillaries (50 μm I.D.) were obtained from Hewlett-Packard.

2.2. Chemicals

All chemicals used for the preparation of the buffers were of analytical-reagent grade (Merck, Darmstadt, Germany) and were used as received. Catalase (M_r 250 000) was purchased from Serva (Heidelberg, Germany), carbonic anhydrase (M_r 29 000), ovoalbumin (M_r 45 000), bovine serum albumin (M_r 66 000), SDS, and acrylamide and N,N′-methylenebis(acrylamide) from Sigma (St. Louis, MO, USA) and the neutral marker, benzene, was from Merck.

2.3. Sample preparation

Catalase solutions containing 0.8 g of catalase purified from bovine liver were prepared with deionized water and filtered through Millipore (Bedford, MA, USA) GS filters of 0.22-μm pore diameter. When indicated, samples were boiled for 10 min with 17.35 or 34.7 m*M* SDS before analysis.

2.4. Capillary electrophoresis

The conditions for electrophoretic analysis were as follows: uncoated fused-silica capillaries, total length 72 cm and separation length 64 cm × 50 μm I.D.; electrolytes, sodium borate–phosphoric acid buffer (pH 7.2) containing, when indicated, 3.47 or 50 m*M* SDS; temperature, 20°C; applied voltage, 25 kV; on-line diode-array detection at 200 nm with 16-nm bandwidth; and injection, hydrodynamic by application of 50 mbar for 30 s at the end of the capillary. Benzene at a concentration of 4% (v/v) in the same diluted buffer was used as a neutral marker [7].

2.5. Polyacrylamide gel electrophoresis

To test the homogeneity of catalase preparation and the dissociation of subunits, 150 μl of the corresponding solution, containing about 6.0 μg protein, were mixed with 75 μl of aqueous glycerol and applied on to 12% polyacrylamide gels [9]. The running buffer was 50 m*M* Tris–glycine (pH 8.3) and at this pH the current generated at 180 mV was about 25 mA at 4°C after equilibration. Staining was performed by using $AgNO_3$ [10]. Carbonic anhydrase (M_r 29 000), egg albumin (M_r 45 000) and bovine serum albumin M_r (66 000) were used as standards.

3. Results

3.1. CZE analysis of native and dissociated catalase

By selecting conditions near those which produced the highest efficiency in the separation of acidic proteins [7], native catalase was resolved as only one peak with a migration time of 6.71 min (Fig. 1A and B). The spectrum of the substance producing this peak showed an absorbance maximum at 280 nm and a secondary peak at 410 nm (Fig. 1C), which revealed the occurrence of the haem iron in the chromoprotein molecule. No dissociation of the protein and no modification of its spectral characteristics were observed when catalase solution was previously boiled with 17.35 m*M* SDS for 10 min before electrophoretic analysis, although the migration time was delayed to 10.48 min (data not shown). However, two different peaks with migration times of 12.18 and 12.29 min were

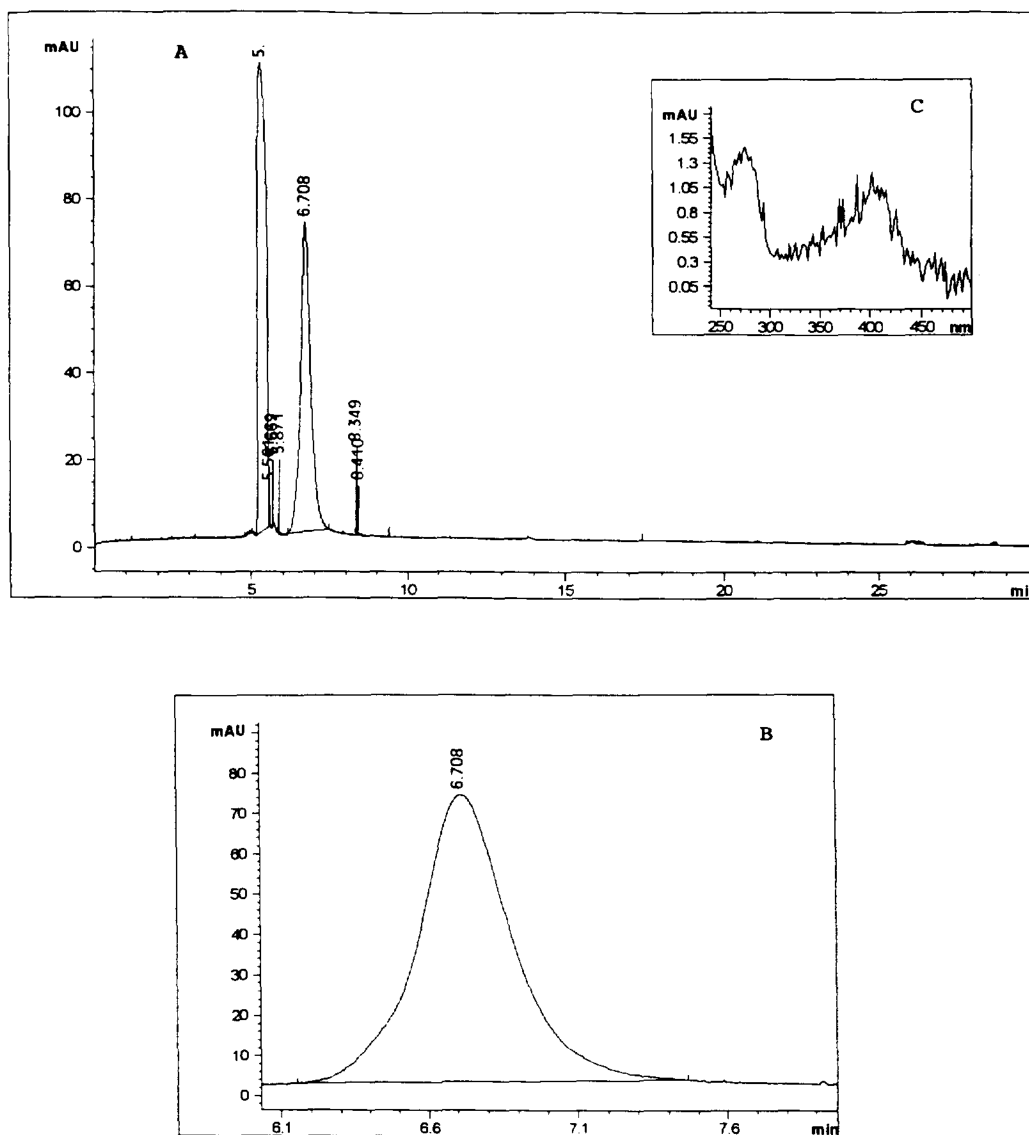

Fig. 1. (A) Normal and (B) amplified capillary electrophoretic profiles of catalase using benzene as neutral marker. (C) Automated absorption spectrum of the protein which moves with a migration time of 6.71 min.

revealed when analysis was performed by using a catalase solution previously boiled with 34.7 m*M* SDS for 10 min (Fig. 2A and B). Absorption maxima at 280 and 410 nm were found for the two peaks, revealing that catalase dissociated into two subunits retaining the haem group after separation (Fig. 2C).

3.2. Effect of modifier and catalase analysis by micellar electrokinetic chromatography

No significant modifications of this elution pattern were effected by including 3.47 m*M* SDS as a modifier in the running buffer (data not shown), although this modifier clearly affected

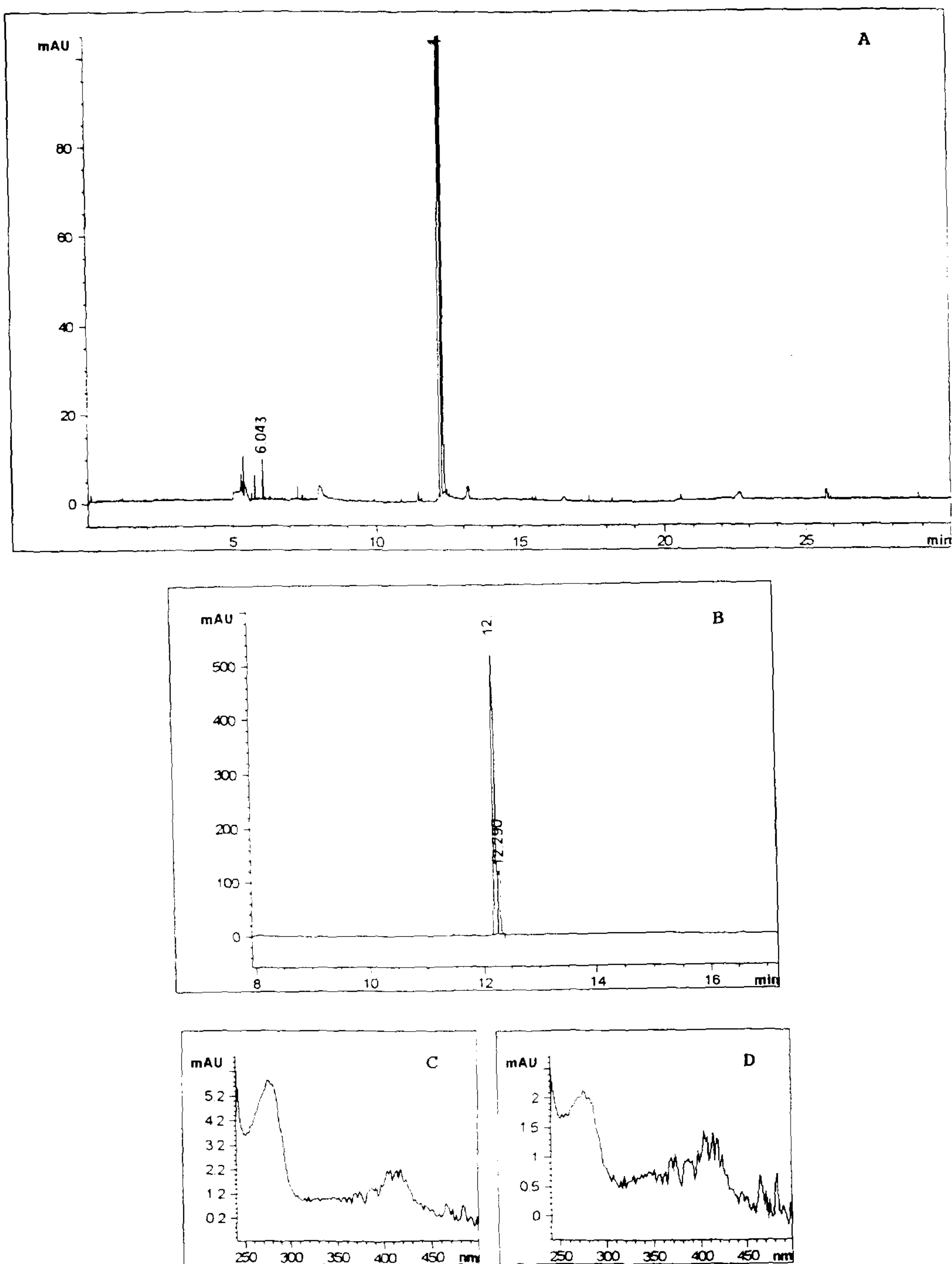

Fig. 2. (A) Normal and (B) amplified capillary electrophoretic profiles of catalase boiled with 34.7 m*M* SDS before analysis, using benzene as neutral marker. (C), (D) Automated absorption spectra of proteins that move with migration times of 12.18 and 12.29 min, respectively.

the broadening of the solute zones on the capillary. However, concentrations of SDS in the range defined for a micellar electrokinetic separation (50 m*M* SDS) added to the running buffer produced two main peaks which separated with an efficiency lower than that shown in absence of the surfactant in the running electrolyte (Fig. 3A and B). Absorption spectra corresponding to these two electrophoretic peaks were characterized by a maximum at 280 nm, although that which should have revealed the haem group appeared considerably distorted (Figs. 3C and D). This maximum was completely lost when catalase was boiled with 17.35 or 34.7 m*M* SDS before the electrokinetic process and, in addition, no separation of the subunits was achieved (Fig. 4). Several peaks different from electric spikes showed spectral characteristics corresponding to degraded protein with maxima displaced towards 265 nm.

3.3. *PAGE and SDS-PAGE of catalase*

Electrophoretic analysis of native catalase in polyacrylamide produced only one band, which dissociated into two well defined bands after boiling the protein with 34.7 m*M* SDS before analysis (Fig. 5, lanes a and b). When separation was carried out using a gel containing 3.47 m*M* SDS, native catalase dissociated into two main bands (lane c) which was repeated when the protein was previously boiled with 34.7 m*M* SDS (lane d) before analysis. The M_r values of these two bands were estimated as about 57 000 and 50 000, respectively. Several minor bands appeared in a similar way to the small peaks in capillary electrophoresis which showed modified spectral characteristics. On this basis, these bands could be interpreted as small peptides derived from protein degradation during SDS treatment.

4. Discussion

The experiments described here demonstrate that CZE can be used to separate different subunits of a polymeric protein avoiding erroneous interpretations about the nature of minor bands that usually appear in conventional SDS-PAGE. Native, non-dissociated catalase has an M_r value of 250 000 and, according to Glauser and Rossmann [11] and Rossmann and Labaw [12], the enzyme is composed of four identical protomers with an M_r of about 60 000. However, capillary electrophoresis separates two different classes of subunits after protein denaturation with 34.7 m*M* SDS (Fig. 2), both containing their corresponding haem group, as revealed from the absorption spectrum obtained from each peak. These results have been confirmed by conventional PAGE of the denatured protein and, in addition, SDS-PAGE reveals that the M_r of these subunits was 57 000 and 50 000, respectively (Fig. 5).

Separation of two classes of catalase subunits has been achieved by denaturing the protein before analysis with 34.7 m*M* SDS (Figs. 1 and 2) or using the surfactant as a modifier in the electrolyte. Although electroosmosis should not, in principle, affect the broadening of solute zones on the capillary [13], electroosmotic flow can modify the time a solute resides in the capillary [7] and then the efficiency and resolution can also be modified as they are related to the flow-rate. In relation to this, adding a surfactant to the background electrolyte can effectively suppress or change the direction of the electroosmotic flow [14] and then modify the efficiency [15]. Indeed, catalase migrates slower in the presence of SDS and the modifier produces significant broadening of the electrophoretic peak (Fig. 3). However, conditions implying micellar electrokinetic chromatography [16], which include 50 m*M* SDS in the running buffer, do not improve the separation of subunits (Fig. 4). As the hydrophobic tails of the surfactant are oriented towards the centre of the micelles, catalase, behaving as a polyanion at pH 7.2 (p*I* 5.4) [8], is probably rejected from the hydrophilic surface of the anionic surfactant and included inside the micelles. Then, protein migrates as an internalized micellar aggregate, without discrimination between different subunits, in the opposite direction to the electroosmotic flow. This effect could be avoided by

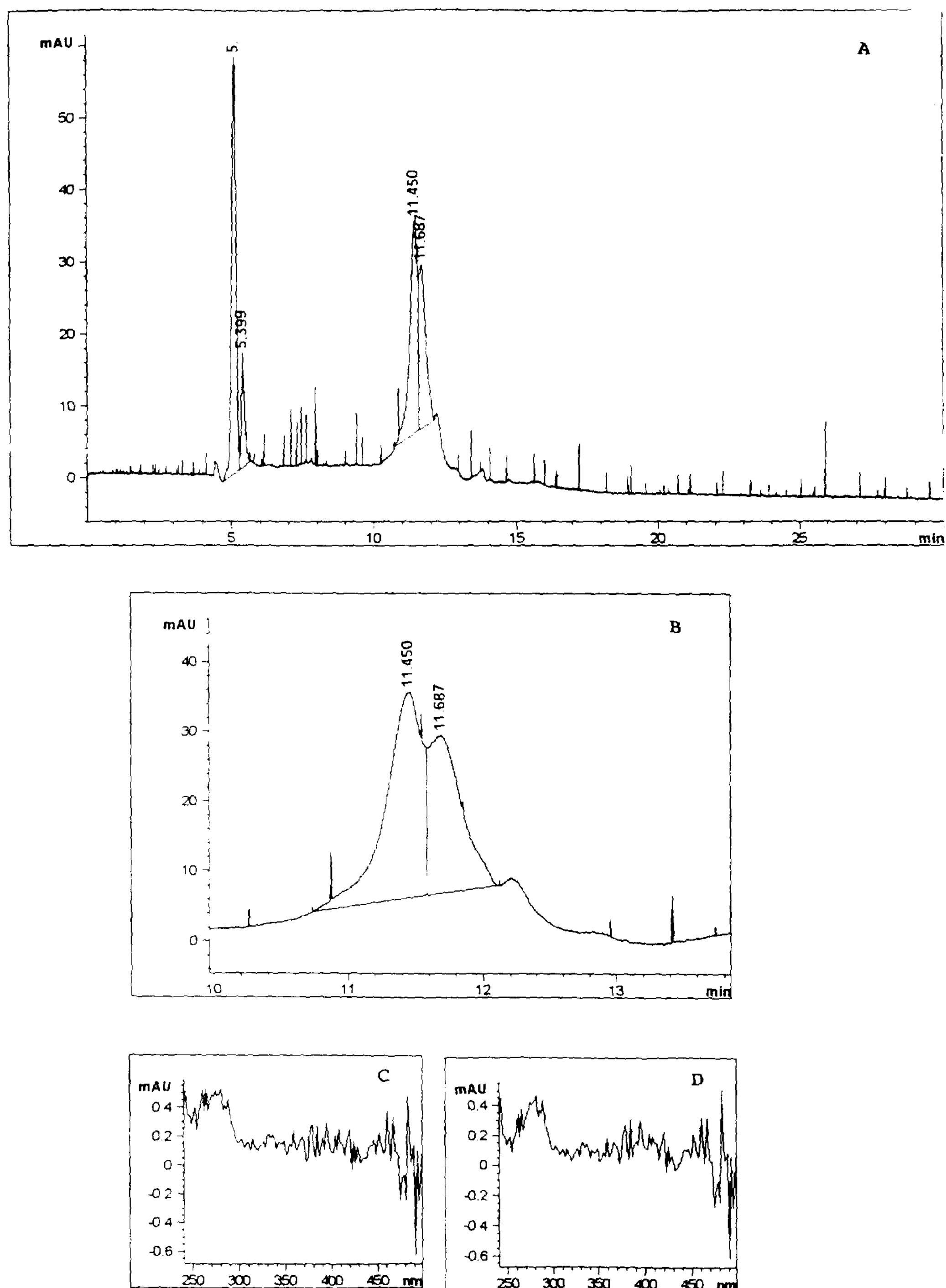

Fig. 3. (A) Normal and (B) amplified capillary electrophoretic profiles of native catalase using benzene as neutral marker and 3.47 mM SDS as a modifier in the running buffer. (C), (D) Automated absorption spectra of proteins that move with migration times of 11.45 and 11.69 min, respectively.

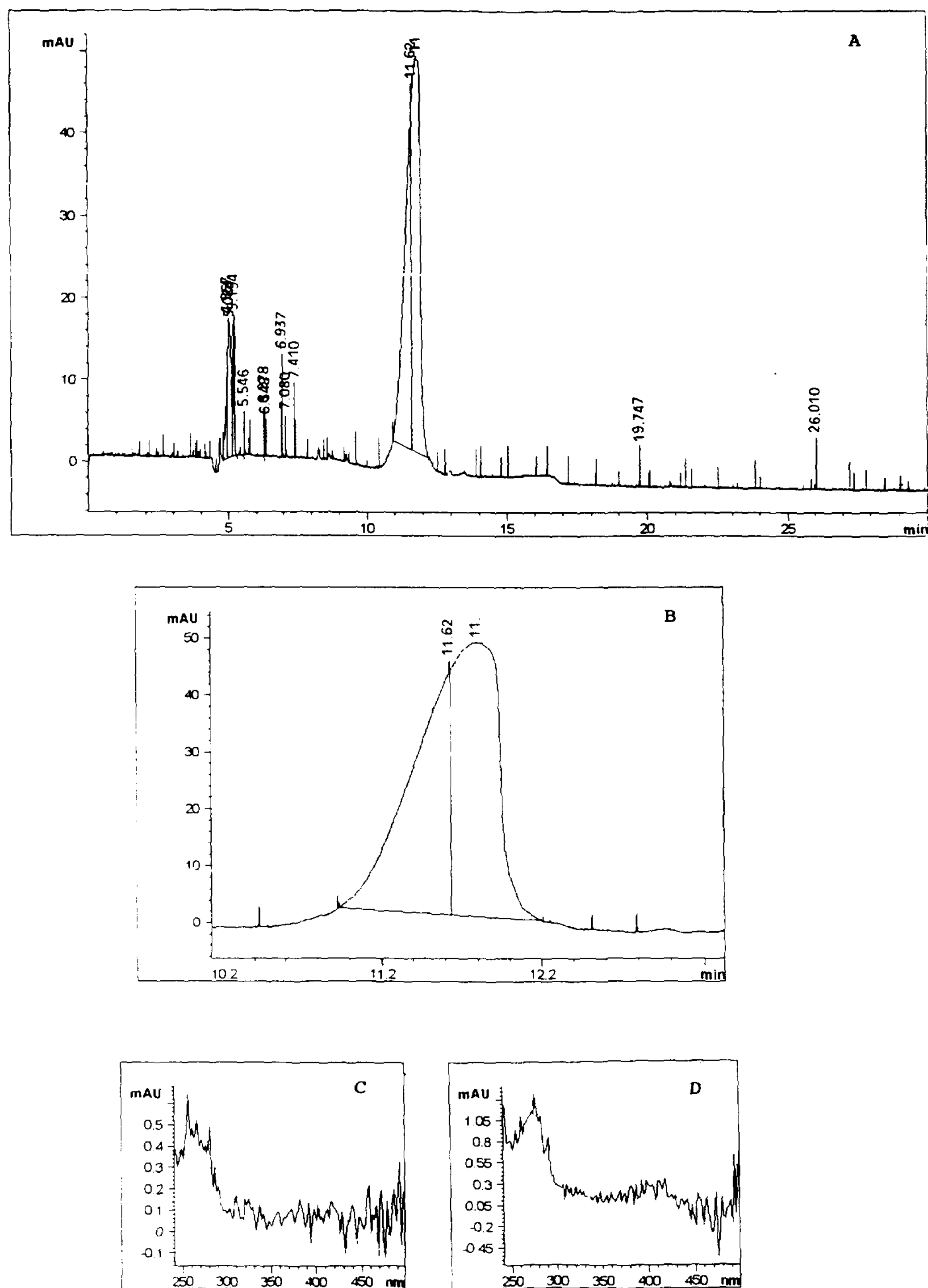

Fig. 4. (A) Normal and (B) amplified capillary electrophoretic profiles of catalase boiled with 34.5 m*M* SDS before analysis using benzene as neutral marker and 3.47 m*M* SDS as a modifier in the running buffer. (C), (D), (E), (F) Automated absorbance spectra of the two zones in which the broadening peak of catalase has been divided.

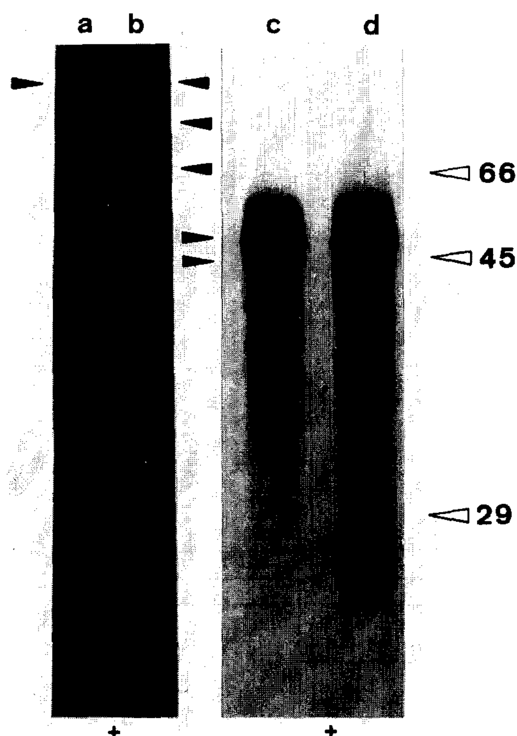

Fig. 5. PAGE of native catalase (lane a) and catalase boiled with 34.7 m*M* SDS (lane b), and SDS-PAGE of native catalase (lane c) and denatured enzyme with 34.7 m*M* SDS (lane d). In SDS-PAGE, the gel contained 3.47 m*M* SDS. Molecular masses of standards, carbonic anhydrase (29 000), egg albumin (45 000) and bovine serum albumin (66 000), are indicated with white arrowheads and catalase and its subunits are indicated with black arrowheads.

using a non-ionic surfactant, such as octylglucoside, complexing with borate [17], but this treatment does not produce a convenient dissociation of catalase.

5. Conclusions

CZE seems to be a very accurate technique for analysing protomers composing polymeric enzymes, such as catalase, when they are previously denatured with SDS. This technique permits the correct identification of the separated subunits and provides arguments to discard some artifactual, degraded subunits which usually appeared in SDS-PAGE analysis. However, MECC with SDS micellar phases exhibits increased retention and a decrease in the efficiency of separation.

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