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A New Approach to Counteracting Chromatographic Electrophoresis

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ABSTRACT

A newly designed apparatus, the J-column, was used to implement the concept of counteracting chromatographic electrophoresis (CACE). The process of separation and focusing was visualized by a model mixture of hemoglobin and ferritin. According to isoelectric focusing analysis, hemoglobin can be purified from the hemolysate mixture by CACE on a J-column.

INTRODUCTION

Counteracting chromatographic electrophoresis (CACE) is a focusing and preparative purification method for charged species and is a powerful separation technique based on the combination of chromatography and electrophoresis (1, 2). Ever since the introduction of the principles of CACE, many attempts and theoretical discussions have been made for its development (3–10). However, the applications of CACE to purify useful compounds from complex mixtures are limited to date.

Previous experimental investigations on CACE more or less followed O'Farrell's column design (2). Briefly, electrolysis chambers are connected to the ends of a chromatographic column through membrane barriers or gel matrices such as a UF membrane, dialysis tubing, or polyacrylamide gel. The convective flow and the electric field are adjusted and maintained to focus

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the charged species of interest at the interface of two different contiguous gel beds. A key point in this process is that the convective flow must be controlled at a stable and extremely slow rate to counteract the electrophoretic movement. In O'Farrell's design, although barriers between the electrolysis chambers and the column may provide some hydraulic resistance to fluid flow, they can not resist the flow absolutely since water molecules pass through these barriers as freely as conducting ions. It is probable that the hydraulic gravity head in the column causes the convective flow rate to exceed the electrophoretic rate or makes the convective flow unstable. Moreover, products of the electrolysis reaction are likely to contaminate the elution buffer through the porous barriers and so change the electromobility of the charged species. As a result, the focusing phenomenon tends to become elusive.

In another line of thinking, Ivory and Gobie proposed a continuous CACE apparatus (9). In this U-shaped column the sample is introduced through a single port in the center of the column and products are withdrawn from the offtake ports continuously. However, this design is suitable only for bicomponent fractionation and no focusing occurs at the gel–gel interface (in fact, there is no gel–gel interface in this design). Products are withdrawn as soon as they are retained below the gel–frit interface. In our opinion, the resolution and practicability of this separation apparatus seem questionable.

Therefore, if CACE is to prove effective and powerful, some modifications to the column design are necessary to address the problems of the existing columns for CACE, and practical preparative purification by CACE should be examined. In this paper a new design for a CACE column is proposed and its performance is reported.

J-COLUMN

This new column design is based on the simple principle that the reaction products of electrolysis could be removed effectively by the supply of elution buffer by which the composition of the buffer in the gel matrices is kept constant throughout the operation process. Also, the electrolysis chambers and the flow field could be integrated without membrane barriers or gel matrices, so that fabrication and operation of the CACE column is simplified. Because this novel apparatus is shaped like the letter J, we call it a J-column.

A J-column consists of three modules: top chamber, separation column, and bottom U-tube (Fig. 1). The top chamber serves as both the buffer reservoir and electrolysis chamber. The sample is loaded onto the glass frit by a syringe through the inlet tube. A buffer containing electrolytes is pumped into the top chamber through the inlet tube. The volumetric flow rate of the buffer is controlled at a level higher than that of the buffer elution rate; thus

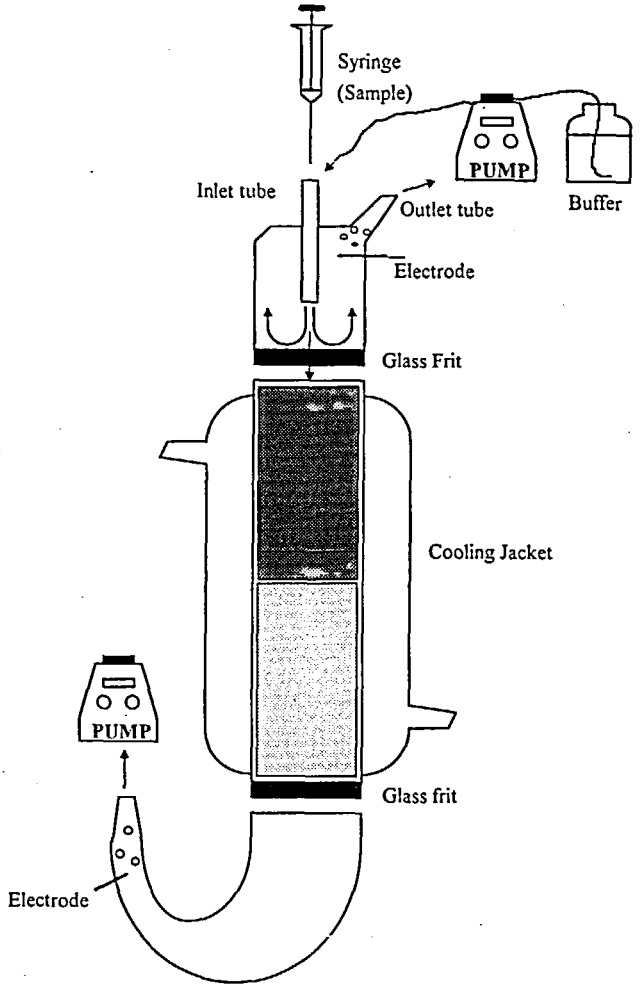

FIG. 1 The architecture of the J-column. It consists of three modules: top chamber, separation column, and bottom U-tube.

an upflow of excess buffer is created. This flow removes the electrolysis products around the electrode through the exit tube. A sintered platinum electrode is fixed near the exit tube. The glass frit in the top chamber acts as 1) a flow distributor which uniformly passes sample and buffer to the gel matrices and minimizes the disturbance in the gel bed, 2) a separation diaphragm which separates the top chamber from the separation column and maintains

the constitution of the buffer in the gel bed during CACE, 3) an ion channel through which electric field passes, and 4) an antifluidization device which maintains the tightly packed configuration of the gel bed during the CACE process.

The separation column is fully packed with two different contiguous gel matrices, i.e., exclusive and inclusive gels. Ice-cold water is circulated in the cooling jacket. The glass frit in the separation column acts as 1) a gel bed support which retains fine gel particles in the separation column, and 2) a separation diaphragm which separates the separation column from the bottom U-tube and prevents electrolysis products from contaminating the buffer in the gel bed.

The bottom U-tube serves as an electrolysis chamber and an effluent tube for the J-column. The sintered platinum electrode is fixed at the side arm of the bottom U-tube opposite the separation column, so that the bubble can be removed steadily with the buffer flow. The convective flow rate is fully controlled by a peristaltic pump at the exit without influencing the hydraulic head of the system.

The dimensions of the J-column used in this research are 13 mm o.d. and 12 mm i.d. The top chamber is 50 mm long and the separation column is 150 mm long. The height of the bottom U-tube is 50 mm.

MATERIALS AND METHODS

Column Preparation

The J-column was packed with an exclusive gel matrix, BioGel P-4 (Bio-Rad), on top of a more inclusive gel matrix, BioGel A-15 (Bio-Rad). The gel bed was equilibrated with the elution buffer overnight, and the cooling jacket was kept at 4°C during the experiment.

Electrolyte Buffer

A 3.9-mM Tris/47 mM glycine buffer adopted from the electrochromatographic experiment by Rudge et al. was used (11). The desired combination of buffering capacity and low ionic strength, and thus low conductivity and minimal heating, is obtained with this buffer.

Target Proteins

Stroma-free hemoglobin was obtained through a simple procedure of hemolyzing the porcine red blood cell followed by centrifugation to remove the cell debris (12). Ferritin was purchased from Sigma Co., USA.

Operating Procedure for CACE on J-Column

Drain the buffer in the top chamber to the level of the frit, and then inject protein solution onto the upper surface of the frit by a syringe and wash it into the gel bed with additional buffer. While the protein solution is being completely carried into the bed, pump fresh buffer into the top chamber and monitor the movement of the protein. Apply an electric field of appropriate magnitude and direction to the column and adjust the effluent flow rate to establish the desired conditions for the protein to focus at the interface. After equilibrium is maintained for a certain period of time, turn off the DC power supply and let the protein flow out of the column.

In this study the fluid flow was controlled by peristaltic pumps (Gilson minipuls-3, France) and the electric field was maintained by a DC power supply (Hoefer Science, USA).

Biochemical Assay

The concentration of hemoglobin was determined from a calibration curve made by Drabkin's method (13). The purity of hemoglobin was determined by isoelectric focusing (IEF3-9, PhastSystem, Pharmacia, Sweden).

RESULTS AND DISCUSSION

Experimental Observations of CACE of Hemoglobin

Figure 2 shows the formation of hemoglobin equilibrium band adjacent to the interface. The experimental conditions for hemoglobin to focus in this equilibrium zone are illustrated in Table 1. In this experiment, 0.5 mg of hemoglobin was loaded into the J-column. After the equilibrium zone was established, a sharply focused band was formed just below the interface, and a diffuse stream as long as 1 cm emanated from the base of the focused band. This phenomenon was also observed for ferritin focusing in the apparatus designed by Ivory et al. (9).

On the other hand, the focusing conditions for hemoglobin were also verified by measuring the counteracting net rate of hemoglobin under different operating parameters, i.e., varying voltage at constant flow rate (1 mL/h) or different flow rate under constant voltage (550 V). As we can see from Figs. 3 and 4, hemoglobin moves downward in the upper phase and upward in the lower phase (i.e., conditions for focusing at the interface) when the flow rate and voltage are set to 1 mL/h and 550 V, respectively.

From our experiences of making CACE experiments on the J-column, we noted that the concentration profile in the focusing region changes as different equilibrium conditions are used. As shown in Fig. 5, the focusing band below the interface looks narrower and denser than in Fig. 2 although the conditions

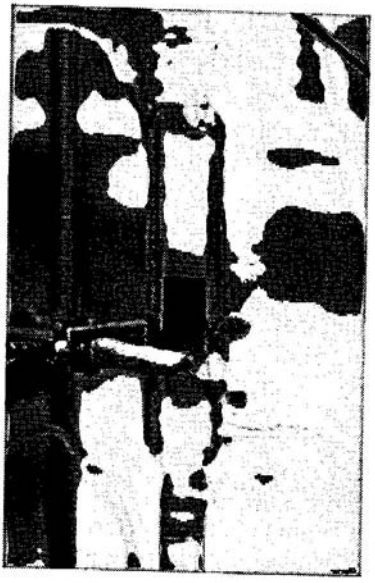

FIG. 2 CACE of hemoglobin on a J-column. A sharply focused band formed below the interface and a diffuse stream emanated from the base of the focused band. This photograph was taken after 8 hours of equilibrium.

are identical with those listed in Table 1 except that the flow rate was slightly reduced (ca. 0.95 mL/h). According to O'Farrell's explanation (1), a concentration-dependent effect causes the electrophoretic rate (R_e) to equal the convective flow rate (R_f) during the focusing process. When the fluid flow rate is reduced and the conditions are still qualified for focusing, i.e., the net movement (R_n) is directed to the interface, the concentration in the focusing band increases further to reduce the electrophoretic rate until the convective rate is canceled (Fig. 6). Obviously, from the viewpoint of focusing, the

TABLE 1
Experiment Conditions for Hemoglobin (0.5 mg) CACE in a J-Column

Electric potential	550 V
Column length	25 cm
Column diameter	1.2 cm
Flow rate	1 mL/h
Buffer	Tris-Glycine, pH 8.3

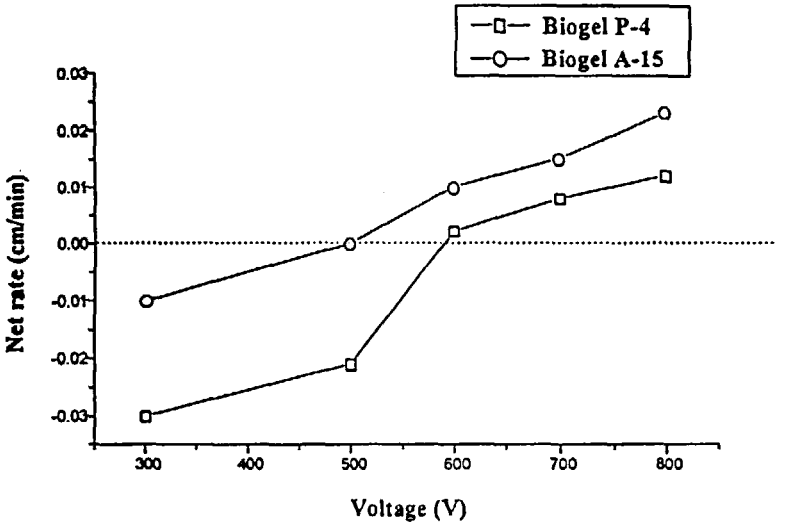

FIG. 3 Counteracting net rate of Hb movement in different gel matrices vs voltage (at constant flow rate, 1 mL/h). The dotted line represents the transition between upward and downward movement.

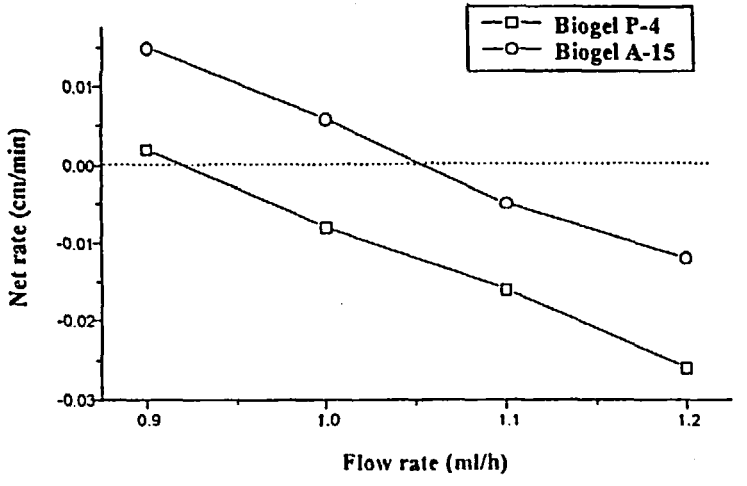

FIG. 4 Counteracting net rate of hemoglobin movement in different gel matrices vs flow rate (at constant voltage, 550 V). The dotted line represents the transition between upward and downward movement.

FIG. 5 A more dense and narrow band was formed when the voltage and flow rate were set to 550 V and 0.95 mL/h, respectively. It seems that the conditions are "edge" conditions for equilibrium (cf. Fig. 4).

conditions for Fig. 5 are preferable to those for Fig. 2. Nevertheless, we found that if the flow rate was reduced a little bit more (<0.95 mL/h), hemoglobin moved upward and the focusing equilibrium vanished. It seems that the conditions in Fig. 5 are the "edge" conditions for equilibrium (see also Fig. 4) and some minute variations in experimental parameters might lead to nonfocusing, which suggests that it is not a favorable choice for operation.

Visualization of CACE Separation Process

To visualize the separation process of CACE, a protein mixture containing hemoglobin (ca. 0.5 mg) and ferritin (ca. 0.5 mg) was loaded to the interface

of the separation column (Figs. 7 and 8). Then the experimental conditions were set to focus the hemoglobin according to Table 1. At first, ferritin moved out of the mixture band little by little ($R_e > R_f$), and once it separated from the band the electrophoretic rate increased due to the concentration-dependent effect. Therefore, the ferritin molecules out of the band migrated upward faster than the ferritin molecules remaining in the band. Gradually, ferritin distributed sparsely over almost all of the upper gel matrix while hemoglobin began to form the equilibrium band below the interface. Finally, ferritin was

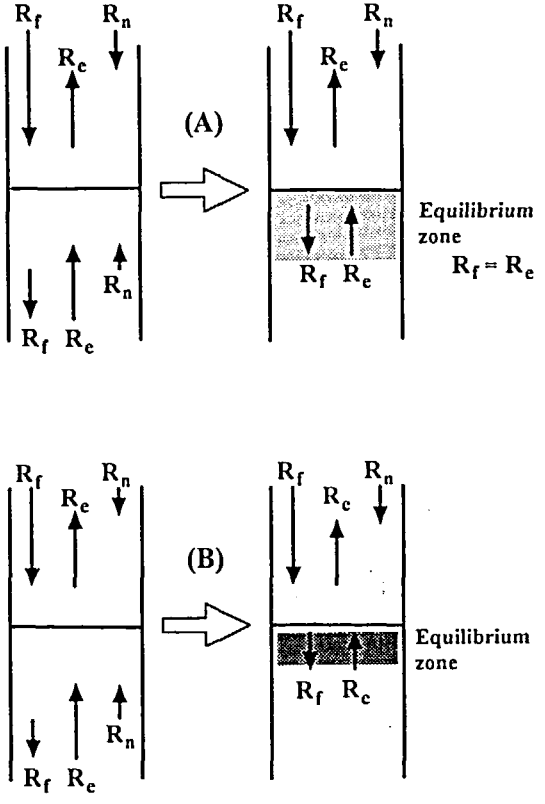

FIG. 6 Schematic explanation of equilibrium formation under different conditions. (A) As charged species move to the interface, the concentration-dependent effect causes the electrophoretic rate to equal the convective flow rate, and an equilibrium zone is formed. (B) If the flow rate is lowered but the net movements are still directed to the interface, the concentration in the equilibrium zone increases further to reduce the elcectrophoretic rate until the convective rate is canceled; thus, a more dense and narrow band is formed.

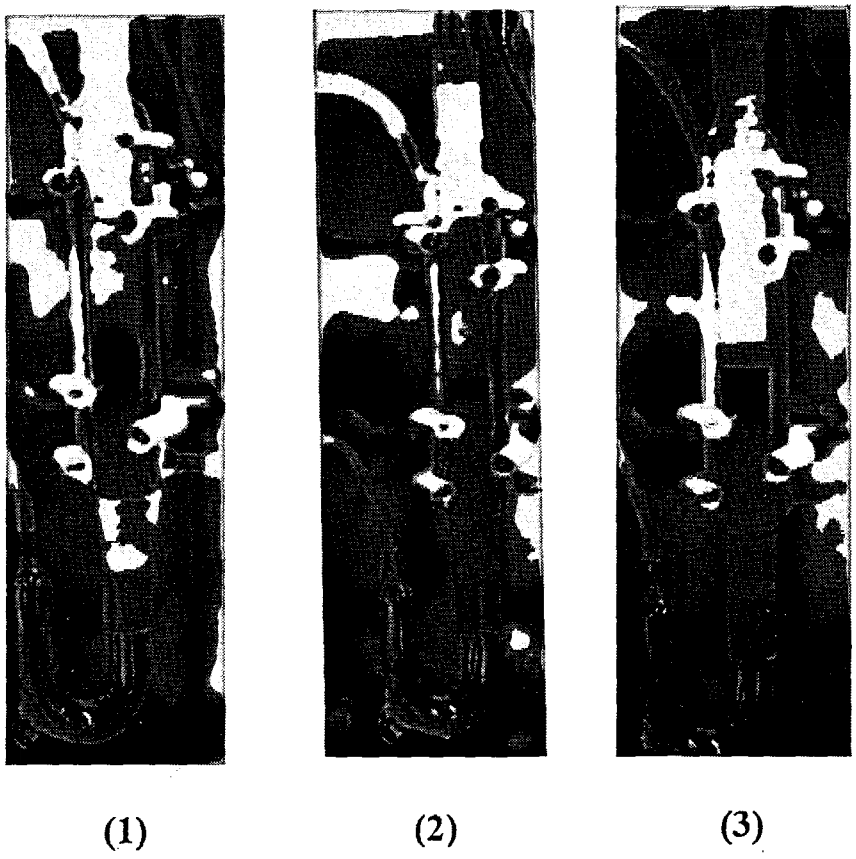

FIG. 7 Visualization of CACE separation. (1) A mixture containing hemoglobin and ferritin was loaded to the interface (0 hour). (2) Hemoglobin focused at the interface while ferritin moved upward (3 hours). (3) Hemoglobin established its equilibrium and ferritin was retained at the top of the gel bed (6 hours).

retained and accumulated below the gel–frit interface because its electrophoretic rate could not exceed the buffer elution rate in the frit.

Purification of Hemoglobin from Hemolysate by CACE

Porcine hemoglobin was selected as the target protein to evaluate the purification power of CACE. Stroma-free hemolysate solution (1 mL) was loaded into the J-column and the experimental conditions were set again to focus hemoglobin according to Table 1. Different samples collected from different

hours of operation were titered to the same concentration (0.5 mg/mL) before analysis for the purpose of comparison. The purity of hemoglobin was identified by iso-electrofocusing (IEF 3–9, silver stain). As shown in Fig. 9(a), impurities in the hemolysate before purification form intensive bands in Lane 4 whereas these bands, except for carbonic anhydrase (pI = 6.3), gradually vanish from Lane 1 to Lane 3 with increased hours of operation. In Lane 3, Hb A, MetHb, and some variants of Hb (F, A2, etc.) form major contiguous bands from pI 6.9 to 7.3. Despite that, carbonic anhydrase (pI = 6.3) was not removed from the hemoglobin solution by CACE under such conditions. However, we think that good separation had been obtained since carbonic anhydrase binds to hemoglobin with a high association constant (14). Various kinds of techniques in the literature also failed to separate carbonic anhydrase from hemoglobin after the purification process (15–17).

Among other analysis results, we found a special case in which hemoglobin was highly purified under the conditions that higher voltage (800 V) was counteracted with a faster flow (ca. 1.3 mL/h) for 10 hours (Fig. 9b). At

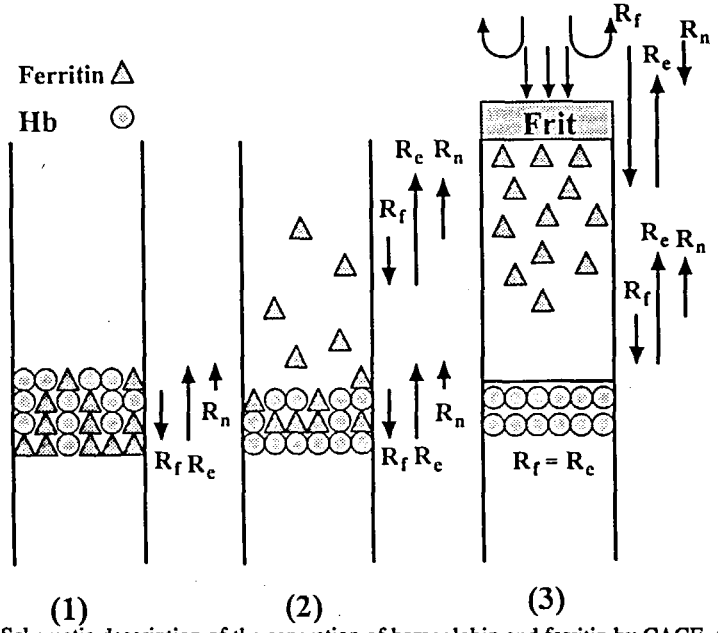

FIG. 8 Schematic description of the separation of hemoglobin and ferritin by CACE. (1) For ferritin, the electrophoretic rate exceeded the convective flow rate. Ferritin moved upward. (2) Once ferritin moved out of the mixture, the electrophoretic rate increased due to the concentration-dependent effect. Ferritin distributed sparsely over the upper gel matrix. (3) Ferritin was retained below the gel–frit interface because of the higher fluid flow rate in the frit. Hemoglobin (Hb) formed a equilibrium zone below the interface.

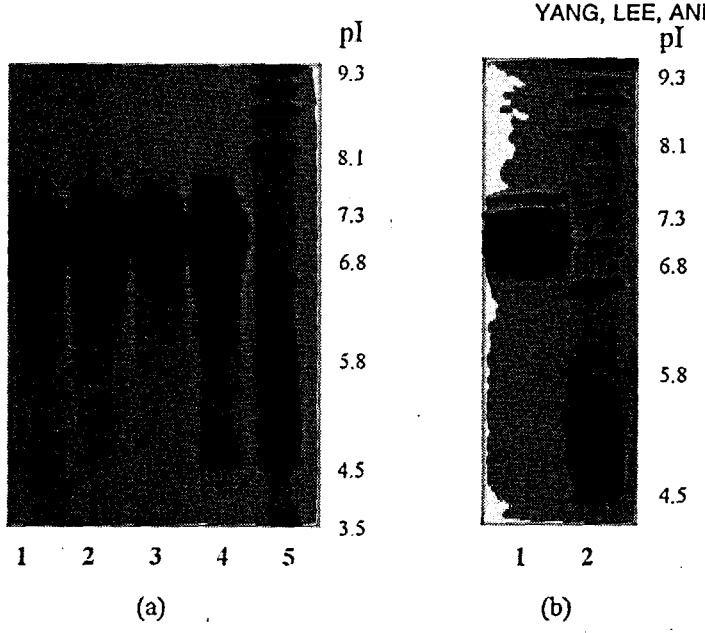

FIG. 9 IEF-PAGE of stroma-free hemolysate. (a) Lane 1: CACE for 2 hours. Lane 2: CACE for 4 hours. Lane 3: CACE for 6 hours. Lane 4: Hemolysate without CACE purification. Lane 5: Standard markers. (b) Lane 1: Highly purified hemoglobin. Lane 2: Standard markers. Components of p*I* calibration kits are amylglucosidase (3.5), trypsin inhibitor (4.55), β-lactoglobulin A (5.2), bovine carbonic anhydrase (5.85), human carbonic anhydrase (6.55), myoglobin (6.85 and 7.35), lentil lectin (8.15, 8.45, and 8.65), and trypsinogen (9.3).

present, the relations between the experimental conditions and the purity level are not fully characterized, but they might imply that the counteracting forces are strong and long enough to dissociate the complex of these two proteins and then form an equilibrium zone of hemoglobin.

CONCLUSIONS

A newly designed apparatus, the J-column, is proposed to implement the concept of counteracting chromatographic electrophoresis. The possible interference and contamination of fluid flow from the electrolysis chamber could be eliminated by the integration of electrodes and fluid flow. The observations of hemoglobin focusing under different CACE conditions revealed that the concentration-dependent effect affects the concentration profile in the equilibrium zone. Model protein mixture separation provided some information about how charged species move and separate from each other during the CACE process. The purification of hemoglobin from a stroma-free hemoly-

sate mixture proved the feasibility of CACE application, although further investigations will be required to obtain the optimal conditions and the desired purity level.

In addition, CACE is by nature a very slow process. Hence, if CACE is applied to practical uses, we believe that the batch-type operation mode will best serve the purpose because duration plays a critical role in the overall process. CACE might be a very worthwhile alternative for some high value products which are difficult to purify by other techniques.

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