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Separation of *Lens culinaris* Hemagglutinin by Forced Flow Electrophoresis

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Abstract

The usefulness and versatility of forced flow electrophoresis (FFE) for the separation of proteins were examined with *Lens culinaris* hemagglutinin (lentil lectins). FFE was operated both in the usual single stage and in a more effective, new double-stage scheme. The dependence of the resolution on the flow velocity through the permeable channel and on the electrical field were analyzed. Increased effectiveness in separation was obtained with various pretreatments such as electrofiltration, dialysis, and centrifugation.

INTRODUCTION

Forced flow electrophoresis (FFE), a method developed in the 1950s by Bier (*1*), is a versatile method which has been used for large-scale electrophoretic fractionation and concentration (*2*), electrofiltration of particulate suspensions (*3*), electroosmotic concentration and desalting of macromolecular systems (*4*, *5*), and electroadsorption (*6*). The method takes advantage of the combined effect of electrophoresis and filtration. This idea has been widely applied to cross-flow electrofiltration (*7*) and electro-ultrafiltration (*8*). The flow characteristics of FFE and its similarities with field flow fractionation (FFF) are analyzed in a companion paper in this *Journal* (*9*).

Aqueous extracts of lentils are a convenient model system for the evaluation of preparative electrophoresis. Lentils contain two isolectins LcH-A and LcH-B which form a complex, with pI's 8.15, 8.65, and 8.45, re-

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spectively, as well as a number of inactive proteins. Hemagglutinin activities of the two lectins are identical and they are immunochemically indistinguishable. Each lectin has a molecular weight of 49,000, and each consists of two identical polypeptide chains (*10–12*). In a previous paper (*13*) the performance of several electrophoretic instruments, but not of FFE, was compared in their ability to purify lentil lectins. The purpose of the present work is to continue the evaluation of electrophoretic methods for their suitability for protein fractionation on a relatively large scale. Chromatography presently dominates this technology, but there are now available a number of different electrophoretic instruments (*14*) which provide alternate means for protein fractionation. Scaling up of electrophoretic methodology requires fractionation in free solutions without the presence of gels or other supporting media.

EXPERIMENTAL

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Pretreatment of Extract

Lentils, 750 g, were soaked at room temperature for 4 h in 3 L TRIS (85 m*M*)/boric acid (293 m*M*) buffer and then blended in a Waring blender for 5 min. The homogenized solution was stored overnight at 4°C and then filtered under vacuum through a nylon filter of 15 μm porosity. After filtration, the retentate was washed twice with 3 L buffer. The filtrate contained 60 mg solids per 1 g solution (dried 2 h at 150°C), pH 7.4, K = 2.8 mS. The filtrate was directly applied to the FFE apparatus for the purpose of electrofiltration, resulting in a filtrate of pH 7.7, K = 1.4 mS. This extract was dialyzed against 5 m*M* histidine. The resulting precipitation was eliminated by centrifugation at 9000 rpm for 30 min, yielding a clear solution of pH 6.5, K = 180 μS.

FFE Apparatus and Mode of Operation

The effectiveness of FFE is based on its combination of electrophoresis and filtration. The apparatus comprises a stack of alternate protein–impermeable membranes and protein–permeable filters, defining narrow channels inserted in parallel between the electrodes. The electrical field is perpendicular to the axial flow direction and can prevent particle deposition at the filter. The Joule heat is dissipated by the process fluid which is recycled through a cooling system.

The application of FFE for purification of lentil lectins is facilitated by the fact that they are the most basic proteins in lentil extracts. In the present work we have used FFE for two purposes: clarification of the crude extract by electrofiltration, as a pretreatment step, and for the final pu-

rification of the lentil lectins from the filtrate. Two FFE schemes were used.

The first one is the usual single-stage FFE, previously described (*2*, *15*). At the beginning, all compartments of the apparatus were primed with the specified buffer, then the feed was changed to the extract. At the same time the voltage was applied and all pumps were switched on. After a volume of extract corresponding to the internal volume of the system was collected, recycling of both the feed extract and the output product was initiated. The recycling product stream was monitored by a UV detector (2138 Uvicord S, LKB, Bromma, Sweden), the printer showing whether the system has reached a steady state or not.

The flow rates of both feed and product were controlled by adjustment of rotating speeds of the gear pumps. Five cells (or 10 compartments) were connected in parallel and the total effective area for each cell was $5.9 \times 16.7 = 98.5$ cm^2. Total inlet flow rate was 44 cm^3/min. Total outlet flow rate through filter membranes was sequentially adjusted and the applied voltage between the electrodes was varied at every flow rate. For each condition, once steady state was reached, the pressure difference between input feed and output product and the current between the electrodes were registered. At the same time, samples were collected and their pH, conductivity, and UV absorption at 280 nm were determined.

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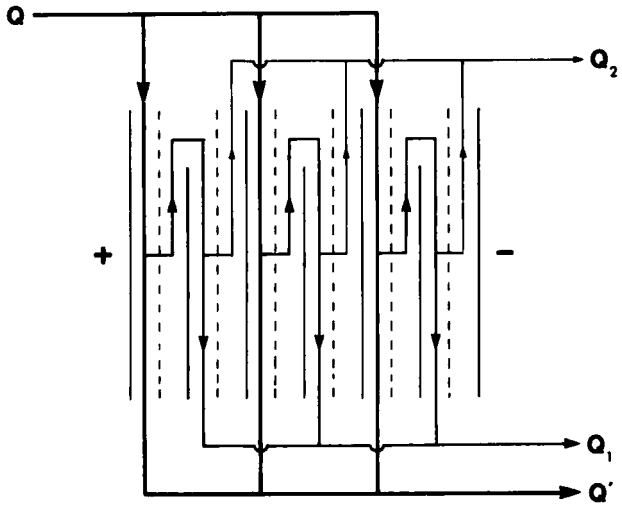

FIG. 1. Schematic representation of double stage FFE. (Q = feed rate, cm^3/min; Q' = residual feed rate, cm^3/min; Q_1 = intermediate product rate, cm^3/min; Q_2 = final product rate, cm^3/min.)

The second scheme is the newly introduced concept of double-stage FFE, shown in Fig. 1. The effluent filtrate of the first stage becomes the feed for the second stage. Each cell has four subcompartments, defined by a membrane, a filter, a cut-down membrane, a second filter and a second membrane, it being shared with the next subcompartment. For the cut-down membrane, located between the second and third subcompartment, the upper one-fifth was cut off. In essence, this imposes within each cell a double-stage FFE, the two stages being separated by the cut-down membrane. The filtrate resulting from the first stage flows through the cut portion of the intermediate membrane and forms the feed for the second stage. Three flows are established: the input feed, the intermediate product derived from the bottom of the second subcompartment, and the final product, the flows being controlled by three gear pumps.

Analysis of the Sample

The concentration of lectins was determined by the agglutination test with fresh rabbit red blood cells, following a standard procedure (*13*). The sensitive but qualitative method of PAGIEF analysis, using Coomassie Blue or silver staining (*16–18*), was also used.

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RESULTS AND DISCUSSION

Pretreatment

Solid debris of ground lentils is not completely eliminated by filtration through nylon mesh and centrifugation at 9000 rpm for 40 min. It may decant to the bottom of the FFE cells and with time inhibit good separation. In addition, with high input velocity and low applied voltage, the debris may clog the filters and render the system unstable. Much improved resolution was obtained by first clarifying the lentil extract by preliminary electrofiltration in the FFE apparatus. Approximately 47 vol% of the original extract was collected. The electrofiltered extract, however, has too high conductivity for efficient FFE due to the salts used in the extraction. Dialysis against water for 24 h in dialysis bags (SPECTRAPOR, MW cutoff: 12,000 ~ 14,000, dry thickness: 0.001 in.) at room temperature about 20°C resulted in discoloration of the extract and flocculation. Conductivity and pH after dialysis were 310 μS and 5.9, respectively. Moreover, both the band of LcH-B and the middle band of the A-B complex have almost disappeared. They presumably have coprecipitated with some of the acidic proteins during dialysis. One could take advantage of this precipitation (*19*); however, we preferred preventing precipitation by dialyzing against 5 m*M* histidine for 45 h at room temperature while maintaining

the pH near 6.5, followed by centrifugation to eliminate the much reduced precipitation. The so dialyzed extract has a low conductivity (180 μS) compared to the electrofiltered extract (1.4 mS). This pretreatment permitted stable operation with a low pressure drop between input and output lines of the apparatus and reasonably high flow rates.

Single-Stage Operation

The operating conditions can be briefly described as follows: Average velocity in the feed channel, $\langle v_x \rangle_0$, at $Q = 44$ cm^3/min was 0.138 cm/s, and the channel Reynolds number based on this velocity and the channel depth (or $N_{Re} = B\langle v_x \rangle_0 / \nu$, where ν is the kinematic viscosity of the solution) was 1.77. The mean velocity through the filter, v_w, in the case of a volumetric flow rate of 5 cm^3/min, was 1.69×10^{-4} cm/s, such that the wall velocity through the filter membrane to the average axial velocity in the channel was 0.00122. The wall Reynolds number, defined as $\mathrm{Re} = v_w B / \nu$, at this velocity was 0.00218.

We first utilized the electrofiltered extract without dialysis as a feed for FFE. Figure 2 shows the Coomassie Blue stained PAGIEF analysis of the

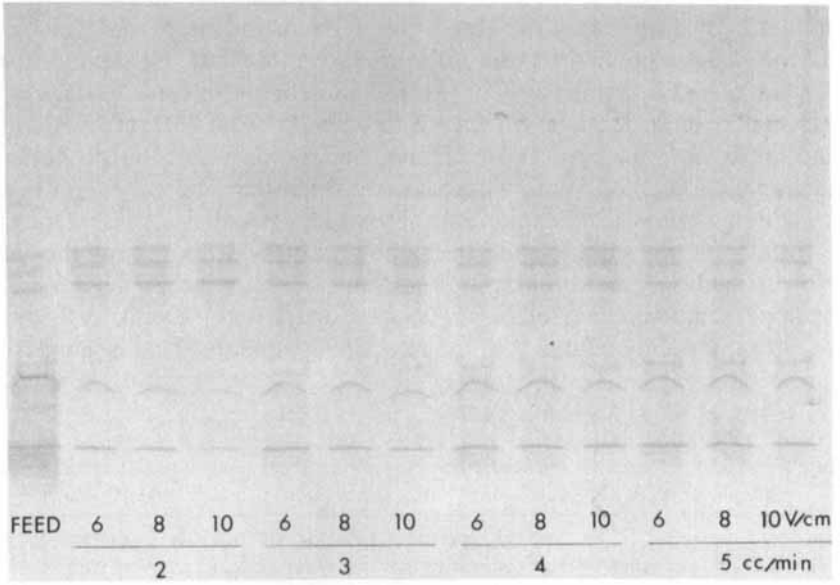

FIG. 2. Coomassie Blue stained PAGIEF analysis in the single-stage FFE with electrofiltered extract. (pH 7.7, $K = 1.4$ mS.)

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recovered fractions. The electric field strength was adjusted sequentially to 6, 8, and 10 V/cm, and the total production rate through the filters was varied from 2 to 5 cm^3/min. At the pH of the extract, 7.7, the lectins are slightly positively charged and will migrate toward the cathode, while most other proteins and particles are acidic and are expected to migrate toward the anode. As can be seen, the filtrate shows the absence or greatly decreased concentration of most of the acidic proteins.

The resolution, as expected, was sensitive to both the applied voltage and the production rate. The resolution itself, however, is not satisfactory, two major acidic proteins remaining in the filtered effluent. To increase the strength of electric field, the voltage was doubled after the conductivity of the solution was decreased through dialysis against 5 m*M* histidine. Moreover, the apparatus was primed with histidine solution of the same concentration instead of the more conductive buffer used before. None of this was sufficient to exclude the two major acidic bands completely. This prompted us to modify the apparatus and introduce the double-stage operation.

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Double-Stage Operation

In spite of the persistence of the main two acidic proteins in the filtered product, we can see that the resolution depends on the output flow rate and the voltage applied. In the following experiment, three double-stage cells were used in parallel, each with four subcompartments. As shown in Fig. 3, the resolution is excellent up to a final production rate of 2 cm^3/min and is not bad even at 3 cm^3/min. Separation at a total production rate (or $Q_1 + Q_2$) of 5 cm^3/min was better than that at 6 cm^3/min (Table 1). The agglutinin activity titer of the extract after dialysis was 256, the activities of intermediates were the same or a little bit more, and the final products had only about half the titer. Since only three cells were utilized in comparison to the five cells in single-stage operation, the velocity through the filter was nearly doubled at the same production rate. That is the reason

TABLE 1
Flow Rates and Potential Gradient (E) Data for Fig. 3. Total Input Flow Rate (Q) was 44 cm^3/min. Lane A Represents Final Products and Lane B Intermediate Products

Lane	1A, 1B	2A, 2B	3A, 3B	4A, 4B	5A, 5B	6A, 6B	7A, 7B	8A, 8B
Q_1 (cm^3/min)	4	3	2	4	3	2	2	2
Q_2 (cm^3/min)	1	2	3	2	3	4	4	4
E (V/cm)	20	20	20	20	20	25	15	15

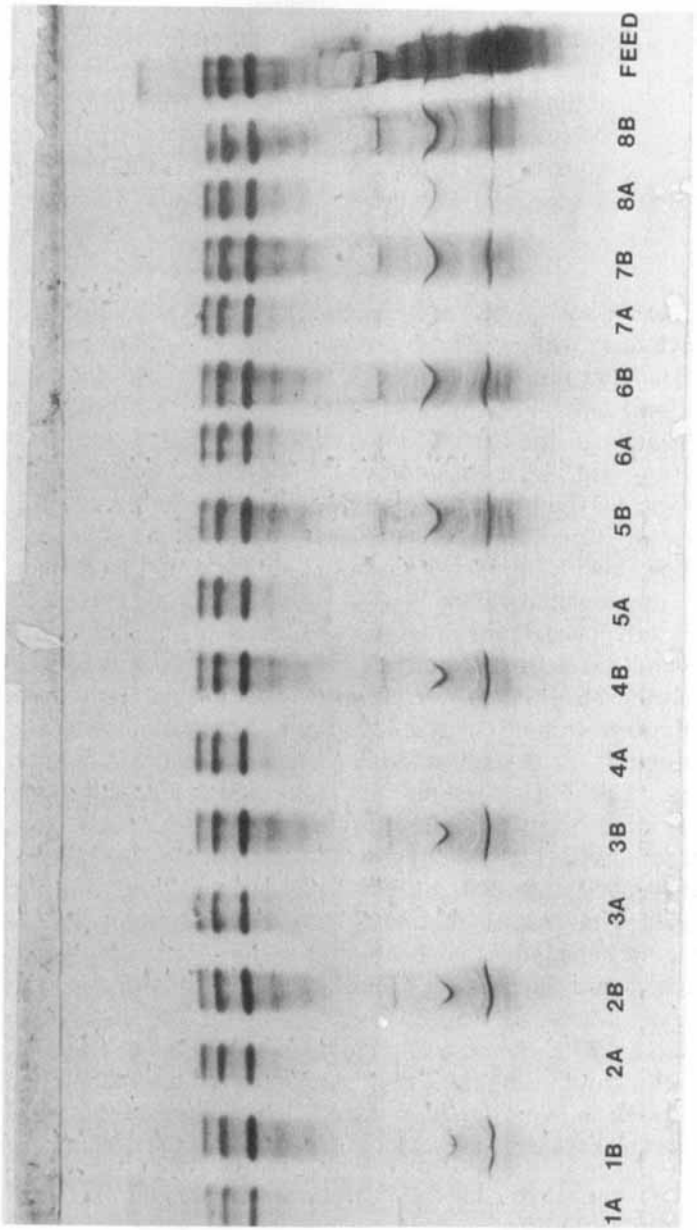

FIG. 3. Silver-stained PAGIEF analysis in the double-stage FFE with electrofiltered, dialyzed extract against 5 m*M* histidine for 45 h. (pH 6.5, $K = 180$ μS.)

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why the intermediate products had a rather high concentration of acidic proteins. With the rest of the extract, continuous bleeding of the final product was done at the final flow rate of 2 cm^3/min to determine the maximum quantity of lectins obtainable. We cut off when 27 vol% of the feed extract was collected, and the product was freeze dried. We harvested 888 mg of dried pure lectins from 750 g of dry lentil, 24 times higher yield than the result with DEAE-cellulose chromatography (*20*).

CONCLUSIONS

FFE was found to be very effective for the separation of lectins from lentil extracts. While we have not optimized any of our procedures, it is quite clear that pretreatments such as electrofiltration, dialysis, and centrifugation render FFE more effective by reducing the conductivity and solids content of the extract. The resolution depends on the voltage and output flow rate, and it was improved in double-stage operation. This mode resulted in very pure lectins. We collected at a flow rate of 2 cm^3/min, continuously, to a total of 27 vol% of the initial feed volume. Extraction could have been continued if a suitable buffer would have been added to replace the depleted volume.

This study has demonstrated the usefulness and versatility of FFE. FFE has several distinctive advantages over other preparative scale biological separators: FFE devices run continuously, and separations are achieved in free solutions without any stabilizing gels. Another advantage is the modular design of the apparatus. Scale-up can be achieved by increasing the number of cells in an assembly. In the past, up to 100 cells in parallel were used. Another way to increase production rate is by expanding the area of the compartments without loss of the resolution. Again, in the past, cells of 100, 500, and 1000 cm cross-section were utilized (*21*). FFE requires only low capital investment and is not expensive to operate. The spacers defining the subcompartments are injection molded, and the nature of the membranes and filters is not critical. Cellulosic dialyzing membranes and a variety of filters can be used.

As shown, FFE can be easily modified to various operational schemes. A model incorporating the essential characteristics of single- and double-stage operation is presented in a companion paper in this *Journal* (*9*). It confirms and explains the greater effectiveness of the double-stage variant.

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