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PURIFICATION OF LENTIL LECTINS USING PREPARATIVE ELECTROPHORESIS

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SUMMARY

Electrophoresis presents an interesting alternative to chromatography for the purification of biological compounds. To document the performance of three preparative electrophoresis apparatus currently available, they were applied to the purification of lectins from lentil seeds which contain two isolectins usually purified by chromatography. Purification by electrophoresis consists of first isolating a mixture of the two isolectins and then separating them. For the first step, either of two free-flow electrophoresis apparatus were employed: the Elphor VaP 22, using field step electrophoresis and the Biostream using zone electrophoresis. To optimize the process, the Biostream was modified to a recycling mode. This required repositioning one dialysis membrane which separates an electrode from the separation chamber. This allowed the lentil extract to be desalted by electrodialysis directly in the apparatus prior to fractionation. A high concentration of lectins was collected at the cathode and acidic proteins were collected at the anode. The bulk of the extract was recycled until the whole volume was processed. In a second step the isolectins were separated by recycling isoelectric focusing in the recycling isoelectric focusing apparatus. The present work clearly demonstrates that electrophoretic methods provide lectins with higher purity than chromatographically purified commercial products.

INTRODUCTION

The seeds of many plants contain proteins called hemagglutinins or lectins. Lectins have been extensively studied for their many interesting properties[1], as they bind specific sugars and agglutinate red blood cells of many animal species. They have also been used to purify glycoproteins. Lectins extracted from the plant seeds are usually purified by chromatography[2–4]. Lentil seeds contain two isolectins LcH-A and LcH-B, differing in charge[3] and isoelectric points (p*I* of LcH-A = 8.15, p*I* of LcH-B = 8.65)[5]. They form a complex[6] which appears in isoelectric focusing gels as a middle band (p*I* 8.45). Each lectin has a molecular weight of 49 kDa and consists of two identical polypeptides. For lentil lectin purification Howard *et al.*[3] used two chromatographic steps: (i) a DEAE cellulose column for the purification of the mix-

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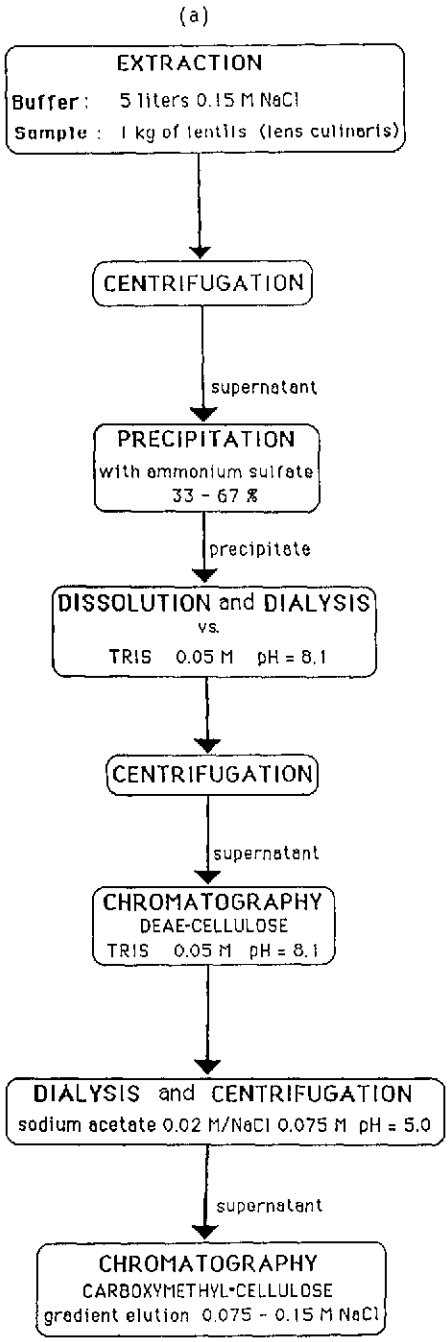
(a)
EXTRACTION
Buffer : 5 liters 0.15 M NaCl
Sample : 1 kg of lentils (lens culinaris)
CENTRIFUGATION
supernatant
PRECIPITATION
with ammonium sulfate
33 - 67 %
precipitate
DISSOLUTION and DIALYSIS
vs.
TRIS 0.05 M pH = 8.1
CENTRIFUGATION
supernatant
CHROMATOGRAPHY
DEAE-CELLULOSE
TRIS 0.05 M pH = 8.1
DIALYSIS and CENTRIFUGATION
sodium acetate 0.02 M/NaCl 0.075 M pH = 5.0
supernatant
CHROMATOGRAPHY
CARBOXYMETHYL-CELLULOSE
gradient elution 0.075 - 0.15 M NaCl

Fig. 1.

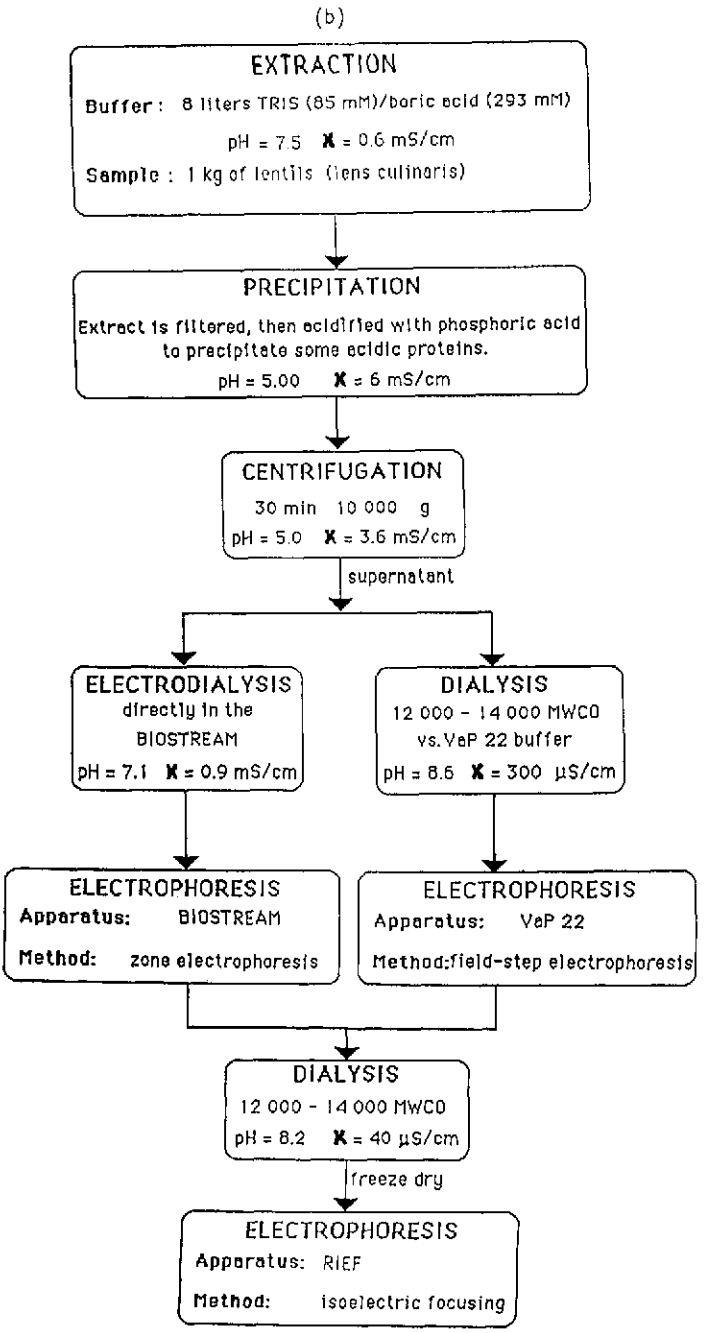

Fig. 1. Schematic representation of lectin purification. (a) Chromatography according to ref. 3; (b) electrophoresis. MWCO = Molecular weight cut-off.

ture of LcH-A and LcH-B and, (ii) a carboxymethyl cellulose column for the separation of the two isolectins. This work shows that the chromatographic steps can be replaced by electrophoretic methods (Fig. 1). The fact that the lectins are the most basic proteins in the extract is clearly an advantage in electrophoretic purification.

Several companies furnish commercial preparative electrophoresis apparatus, three of which were used in this work: the Elphor VaP 22[7], the Biostream[8] and the recycling isoelectric focusing (RIEF) developed at our center[9]. These apparatus differ in throughput, field of applications and operating principles. Free-flow electrophoresis can be operated in several modes: zone electrophoresis (ZE), isoelectric focusing (IEF), isotachophoresis (ITP) and field step electrophoresis (FSE)[10]. An advantage of electrophoresis is its ability to concentrate the sample during purification. For this purpose, FSE and IEF were used in the Elphor VaP 22 and in the RIEF, respectively, while zone electrophoresis was used in the Biostream. Usually ZE dilutes the sample, but our modification of the Biostream to a recycling mode allowed the simultaneous purification and concentration. The isolation and separation of lentil lectins provided a suitable means for comparing these devices. The isolation of a mixture of the two isolectins was accomplished using either the Elphor VaP 22 by FSE or the Biostream by recycling ZE. The separation of isolectins LcH-A and LcH-B was carried out by IEF in a narrow pH profile using the RIEF instrument. The lectins were analyzed by UV absorption at 280 nm, agglutination activity and analytical polyacrylamide gel isoelectric focusing (PAGIEF) and compared to commercially available material. Performance of the instruments was assessed in terms of throughput and purity.

EXPERIMENTAL

Materials

Ampholine carrier ampholytes for preparative and analytical IEF were purchased from LKB Produkter (Pleasant Hill, CA, U.S.A.), acrylamide, Bis, N,N,N′,N′-tetramethylethylethylenediamine (TEMED), ammonium persulfate and urea from BioRad (Richmond, CA, U.S.A.), β-alanine, Coomassie Brilliant Blue G-250, 5-aminopentanoic acid (EACA), Gly-Gly, imidazol, morpholinoethanesulfonic acid (MES) and Tris from Serva (Westbury, NY, U.S.A.), lentils (lens culinaris) from a local food store, LcH-B from ICN ImmunoBiologicals (Lisle, IL, U.S.A.), Lectin from Sigma (St. Louis, MO, U.S.A.), and Vector Labs. (Burlingame, CA, U.S.A.) and reagents for color silver stain from Pierce (Rockford, IL, U.S.A.). All other chemicals were reagent grade.

Lectin extraction

Dried lentils were soaked overnight at 4°C in a Tris (9 g/l)–boric acid (18 g/l) buffer, pH 7.4 and conductivity of 0.6 mS/cm (1 kg lentil seeds in 2.4 l buffer). The seeds were then homogenized in a Waring blender with 1.6 l of the buffer, for 5 min. This suspension was stirred for 1 h at room temperature and then filtered through a nylon screen with 0.5 mm pores. The retentate was washed twice with 2 l portions of buffer and the filtrates were pooled. This filtrate contained 43 g total dry lentil solid/l. It was titrated to pH 5.0 with phosphoric acid (85%, w/v) which precipitated some acidic proteins. The solution was stored overnight at 4°C and then centrifuged at 13 000 *g* for 30 min. A clear solution with a conductivity of 3.6 mS/cm was obtained

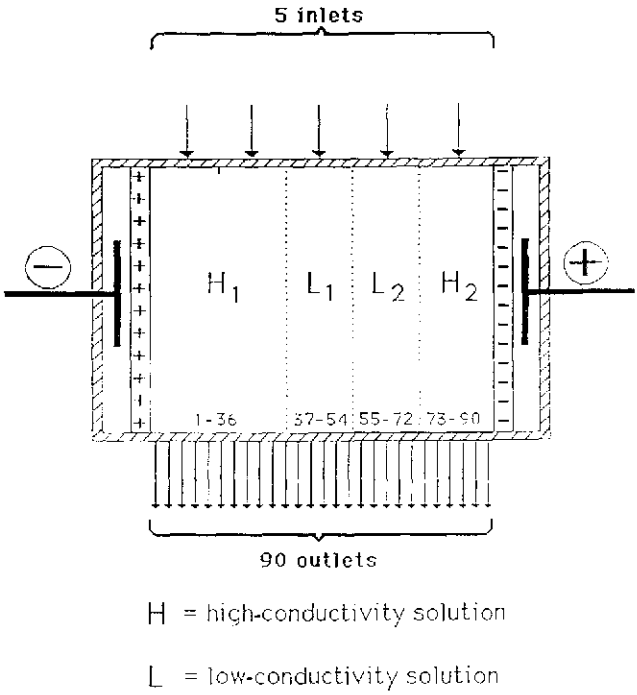

Fig. 2. Elphor VaP 22 principle for field step electrophoresis showing the arrangement of the various buffers. H1 = Catholyte; H2 = anolyte; L1, L2 = sample zones.

and contained 15 g total dry solid/l. The solution was then desalted by electrodialysis or dialysis before electrophoresis.

Electrophoretic apparatus and experimental methods

FSE in the Elphor VaP 22. The Elphor VaP 22 is a continuous free-flow apparatus from Bender & Hobein (Munich, F.R.G.) in which solutions flow between two parallel glass plates. The electrical field is applied perpendicularly to the liquid flow. The solutions are introduced at the top of the chamber through five inlet tubes. Charged proteins are deflected by the electrical field and are collected at the bottom through 90 outlet tubes (Fig. 2). The dimensions of the separation chamber are 100 × 500 × 0.5 mm[7]. This apparatus can be used for ZE, IEF, ITP and FSE[11-13].

For FSE[14,15] the electrolyte system schematically illustrated in Fig. 2 was used. Two solutions of low conductivity (K), one containing the sample, were bracketed between an anolyte and a catholyte of at least 20 times higher conductivity, forming a stepwise conductivity profile. Charged proteins in the sample area migrate electrophoretically until they reach the high conductivity boundary, where their velocity decreases due to the lower electric potential. Thus, they are concentrated at the conductivity interfaces. Two variants of this principle were employed.

Recycling zone electrophoresis in the Biostream. The Biostream is a continuous free-flow apparatus developed at the Harwell Laboratory of the United Kingdom Atomic Energy Authority and commercialized by CJB Developments (Portsmouth,

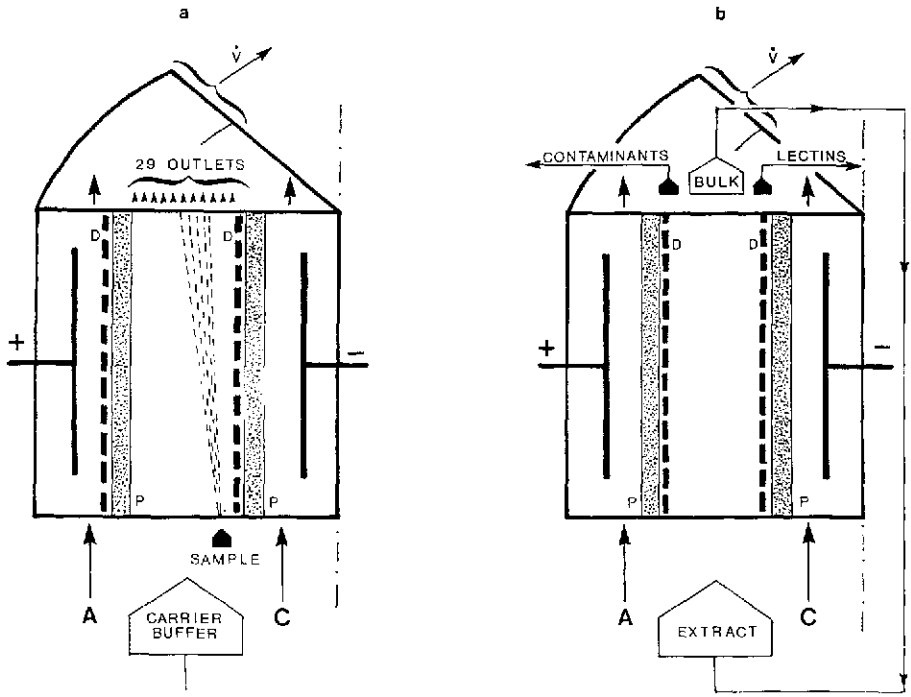

Fig. 3. Schematic representation of the Biostream illustrating the position of the dialysis membranes and the flow paths for (a) the usual configuration as designed by the manufacturer and (b) the recycling mode where lectins and contaminants are continuously withdrawn. A = Anolyte; C = catholyte; D = dialysis membrane.

U.K.). In this machine the electrophoretic separation takes place radially in an annulus between two concentric cylindrical electrodes. The chamber is 1 m in length and 3 mm in thickness. To stabilize the laminar flow, the outer cylinder rotates at 150 rpm during electrophoresis[8]. The samples are collected through 29 outlet tubes at the top of the chamber. The separation chamber is separated from the two electrode compartments by dialysis membranes supported by rigid cylindrical porous spacers. In the usual configuration of the Biostream these membranes are stretched over the external surface of the spacers. The boundaries of the separation chamber are then a dialysis membrane at the inner cylinder (cathode) and a porous material at the outer cylinder (anode). A carrier buffer flows through the chamber from the bottom to the top. The sample is introduced separately through a narrow slit at the bottom of the inner cylinder (Fig. 3a). The separation conditions have to be selected to prevent proteins from reaching and penetrating the outer porous spacer. To avoid this problem, a dialyzing membrane was streched over the inside of the outer spacer (Fig. 3b). As a result, proteins can migrate towards both membranes. The advantages are that the sample solution functions as the carrier buffer and the most acidic and basic fractions are concentrated at the membranes. This is achieved by recycling the sample through the chamber and continuously drawing off proteins that concentrate at the inner and outer membranes. In addition, this allows the use of the Biostream as an

electrodialyzer for preliminary desalting: the solution is recycled until the correct pH and conductivity are reached. For the present work, the fractions closest to the cathode containing pure lectins were collected. The anodic fractions containing acidic proteins were discarded to avoid their accumulation in the chamber. The middle fractions were recycled until the whole was processed. This method is called recycling zone electrophoresis.

Isoelectric focusing in the RIEF apparatus. In the RIEF-apparatus, developed in our laboratory and commercialized by Ionics (Watertown, MA, U.S.A.) the protein and carrier ampholyte solution to be focused is recirculated through a multicompartment apparatus with the ten subcompartments, separated by nylon screens (pore size 6 μm)[9]. The electrical field is applied perpendicularly to the screens and generates the pH profile by focusing of carrier ampholytes[16]. Proteins migrate through the screen separators until they reach a compartment with the pH of their isoelectric point[17,18].

Analysis

The pH and K values of all fractions were measured and the lectins quantified by A280 and agglutinating activity. In addition, analytical IEF gels were utilized as a measure of purity.

Activity tests. The activity of lectins was measured on microtiter plates by serial dilutions of 25 μl of sample with 25 μl phosphate buffer–saline (PBS) (0.05 *M* sodium phosphate–0.15 *M* sodium chloride, pH 7.0)[1,19], added to 25 μl of a 4% rabbit red

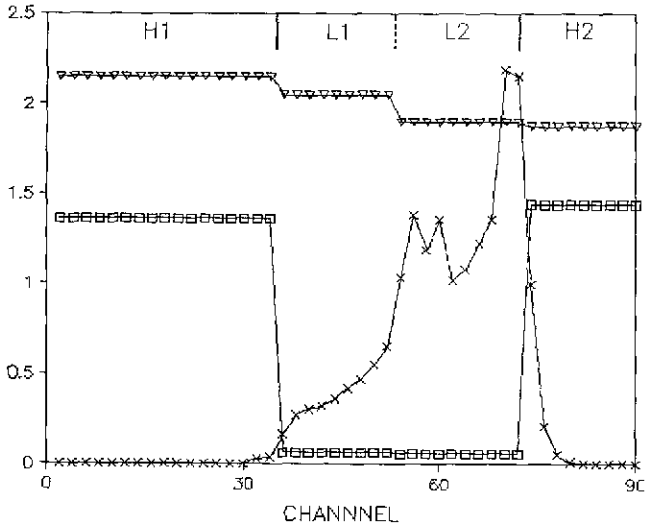

Fig. 4. Lectin purification in the Elphor VaP 22 with sample introduction through L1. The initial pH (▽) and conductivity (□) profiles are shown together with the absorbance measurements at 280 nm (×). Buffer system: catholyte H1: 0.05 *M* phosphate buffer (pH = 8.6, K = 6880 μS/cm); sample L1: lentil extract, dialyzed, in 0.1 *M* imidazole–0.1 *M* EACA (pH = 8.2, K = 314 μS/cm); L2: 0.1 *M* imidazole–0.1 *M* Gly-Gly (pH = 7.6, K = 280 μS/cm); anolyte H2: 0.05 *M* phosphate buffer (pH = 7.5, K = 7200 μS/cm). Separation conditions: current, 125 mA; voltage, 519 V; residence time, 4.7 min; flow-rate per inlet, 65 ml. (Actual pH = pH × 0.25. Actual K = K × 0.0002.)

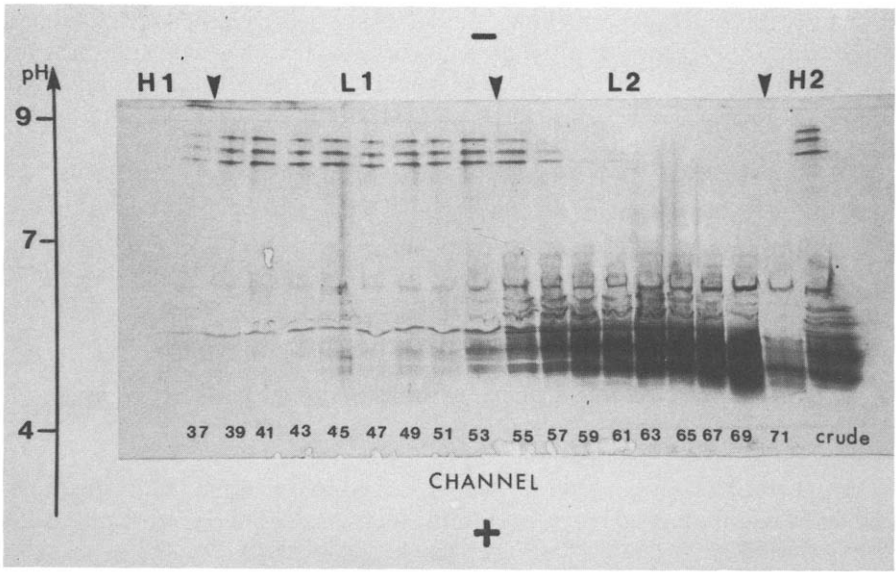

Fig. 5. Silver stained PAGIEF analysis of selected fractions from the experiment of Fig. 4 showing the distribution of proteins along the electrophoresis chamber. The three bands in the basic region are the two isolectins LcH-A (pI 8.15), LcH-B (p*I* 8.65) and a complex of the two forms (p*I* 8.45).

blood cell suspension (40 μl of packed red blood cells diluted to 100 μl with PBS). The highest agglutinating dilution is reported as the activity titer of the solution.

Analytical IEF gel. Native PAGIEF was performed as previously described[20]. Proteins were visualized by Coomassie Blue or silver staining[21,22].

RESULTS AND DISCUSSION

Purification of the lectin mixture

FSE in the Elphor VaP 22. Two different systems of field step electrophoresis were utilized for the lectin purification.

(i) The dialyzed lentil extract was introduced at the cathodic side of the sample area Fig. 4, (L1). Figs. 4 and 5 show the results when the crude extract was dialyzed and adjusted to pH 8.2 with imidazole–EACA. At this pH the lectins are virtually immobile in the sample area L1 and exit the chamber almost without deflection. The acidic proteins of the extract migrate from the L1 area into the low conductivity buffer L2 (imidazole–Gly-Gly, pH 7.6). Lectins which reached L2, reversed direction and returned to the L1–L2 boundary. The acidic proteins at pH 7.6 migrated in the direction of the anode and concentrated at the L2–H2 boundary. In comparison to the initial solution (activity titer: 128) with this method the lectins were purified but not concentrated (activity titer: 64)

(ii) The extract was introduced into the anodic side of the sample area (L2) at an acidic pH, forcing the lectins to the cathode. Figs. 6 and 7 show the results obtained when the crude extract was dialyzed and adjusted to pH 5.99 with EACA – Gly-Gly. This pH was 2.5 pH units below the p*I* of the lectins. When the lectins reached L1 the

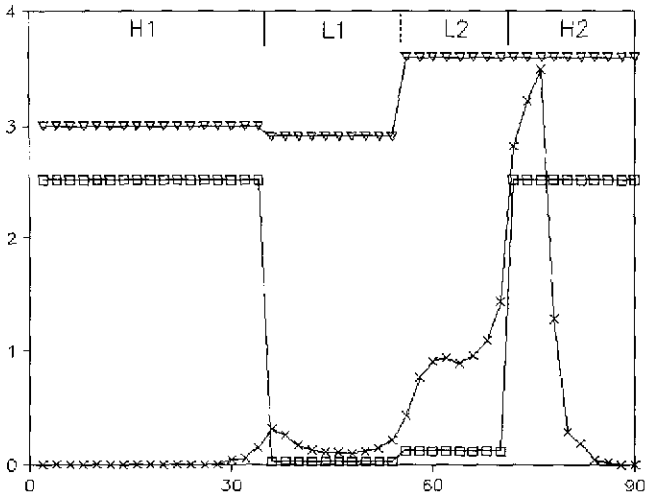

Fig. 6. Lectin purification in the Elphor VaP 22 with introduction of the sample through L2. The pH (▽) and conductivity (□) profiles are shown together with the absorbance measurements at 280 nm (×). Buffer system: catholyte H1: 0.05 *M* phosphate buffer (pH = 5.0, K = 6300 μS/cm); sample L1: 0.1 *M* MES/0.1 *M* β-alanine (pH = 4.85, K = 70 μS/cm); L2 : lentil extract, dialyzed, in 0.1 *M* EACA–0.1 *M* Gly-Gly (pH = 5.99, K = 303 μS/cm); anolyte H2: 0.05 *M* phosphate buffer (pH = 6.0, K = 5800 μS/cm). Separation conditions: current, 200–150 mA; voltage, 1100–1400 V; residence time, 4.6 min. (Actual pH = pH × 0.6. Actual K = K × 0.0004.)

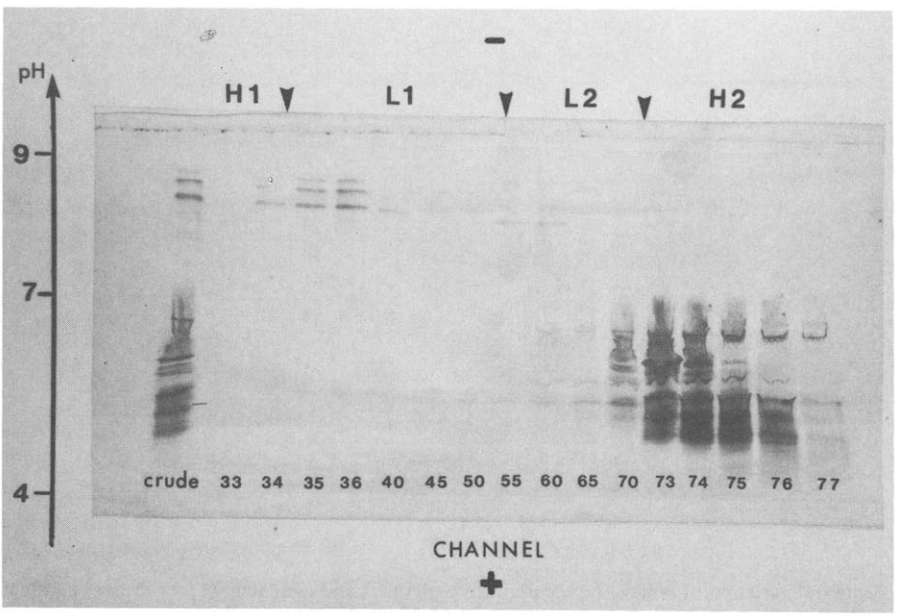

Fig. 7. Silver stained PAGIEF analysis of selected fractions from the experiment of Fig. 6. The activity titers were 128 for the crude extract, 16, 64, 128, 128, 32, 16, 32, 32, 8, 4, 8, 2, 0, 0, 0 for the respective fractions.

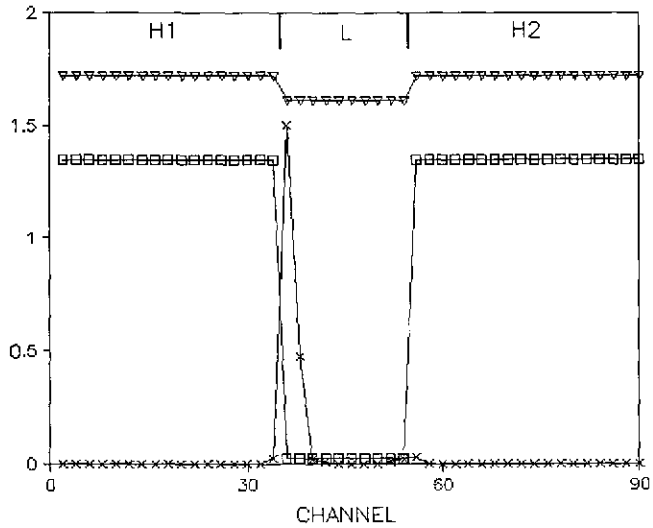

Fig. 8. Lectin concentration by field step electrophoresis in the Elphor VaP 22. The initial pH (▽) and conductivity (□) profiles are shown together with the absorbance measurements at 280 nm (×). Buffer system: catholyte H1: acetic acid–sodium hydroxide (pH = 4.91, K = 6700 μS/cm); sample L: fraction 33–41 of Fig. 6 (pH = 4.6, K = 127 μS/cm); anolyte H2: acetic acid–sodium hydroxide (pH = 4.91, K = 6700 μS/cm). Separation conditions: current, 175 mA; voltage, 630 V; residence time, 3.0 min; flow-rate per inlet, 100 ml/h. (Actual pH = pH × 0.35. Actual K = K × 0.002.)

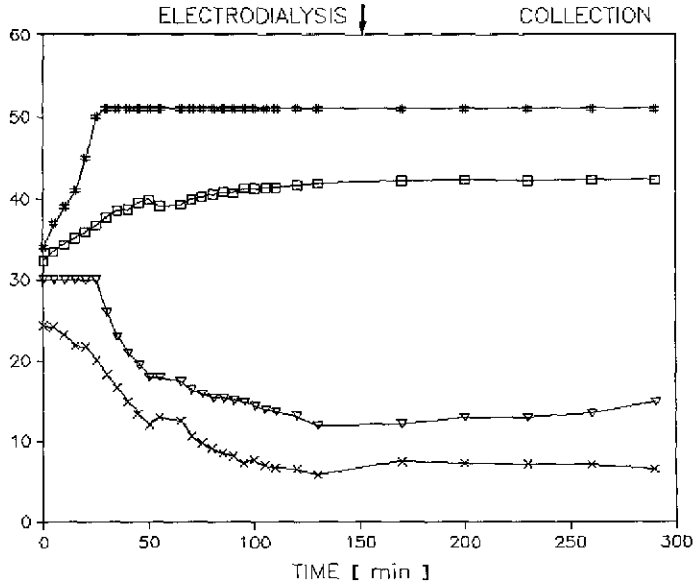

Fig. 9. Temporal variation of voltage (#), current (▽), pH (□) and conductivity (×) of the bulk fluid during electrodialysis and collection of 5 l of lentil extract (pH = 5.0, K = 5.8 mS/cm) using the Biostream in the recycling mode. Electrolyte system: catholyte: boric acid (2 g/l)–sodium hydroxide (pH = 8.0, K = 3.3 mS/cm, flow-rate = 150 ml/min); anolyte: tris (2 g/l)–sodium hydroxide (pH = 5.0, K = 5.0 mS/cm, flow-rate = 150 ml/ min). (Actual pH = pH × 6. Actual K = K × 6.8.)

lower pH of this buffer (MES–β-alanine, pH 4.85) they were accelerated to the field step between L1 and H1. Most acidic proteins stayed in L2 or migrated to H2 (Fig. 7). In this experiment 65 ml/h of dialyzed crude extract with a total protein concentration of 3.3 g/l containing 0.3 g/l of lectins (activity titer 128) were processed. After electrophoresis, lectin activity was detected in fractions 32–74, but only fractions 32–54 contained pure lectins. The recovery rate in fractions 32–54 was 80 ml/h of 0.16 mg/ml or 13 mg lectin/h. In comparison to Fig. 5 it is shown that these lectins have the same activity as the initial solution (activity titer 128). However the specific activity (ratio between activity titer and total protein concentration in g/l shows a 20-fold enrichment (ratio between final and initial specific activity). Fractions 55–74 with 6 mg of lectins and all acidic proteins were discarded. The recovery was 68%.

In a second step, a normal field step system was used to concentrate the purified fractions. The fractions 33–41 (Fig. 7) with the highest lectin concentration were pooled producing 32 ml of lectin solution with 0.2 g/l (activity titer 128) representing 35% of the lectin in the starting material. This solution was introduced at pH 4.6 directly between two high conductivity solutions (Fig. 8), with a flow-rate of 100 ml/h (20 mg lectins/h). The lectins concentrated in only five fractions (35–39), with 80% (16 mg/h) in fraction 36 and 37, seven times concentrated (c = 1.4 g/l, activity titer 1024). The solution was concentrated but not enriched (same specific activity). In summary 16 mg of lectins were produced in 4 h: 3 h for the first step to produce 100 ml of initial pure material which concentrated in 1 h during the second step.

Recycling zone electrophoresis in the Biostream. Five liters of crude extract (4 g total protein/l, activity titer 128, pH 5.0, K = 3.6 mS/cm) were electrodialyzed for 2½ h to pH of 7.02 and K 0.8 mS/cm. The final current was 10 A, the final voltage, 50 V (Fig. 9). The extract was recycled at 1 l/h through the chamber. The distribution of lectins across the chamber (29 fractions) at this state is shown in Fig. 10. Then fraction 1, containing pure lectins, was collected at 210 ml/h. The contaminants were removed from fraction 29, at the same flow-rate. The activity titers of these fractions was 512 and 128 respectively. The total protein concentration of fraction 1 was 1.3 g/l, determined by dry solid weight after dialysis against water. Fig. 11 presents a comparison of the purity obtained with the Biostream to commercial lentil lectins purified by chromatography.

This purification by recycling ZE was performed as a batch process. Thus, the concentration of collected lectins decreased with time (for example from 2.2 tot 0.55 g/l). The extract was processed for 5 h in the Biostream. After this time, the lectin concentration in fraction 1 decreased to 1.3 g/l, similar to that obtained with the VaP 22. During this time, 1 l of lectins solution (1.3 g/l) was produced, representing an average of 250 mg of lectins/h and a yield of 56%. This represents an about 60 times higher processing rate than with the VaP 22 with a comparable recovery and enrichment.

Separation of LcH-A and LcH-B by isoelectric focusing in the RIEF

To separate the two lectins, (pI difference of 0.5 pH units), two sequential RIEF steps were performed (Fig. 12).

(i) A volume of 270 ml of lectin solution, prepurified in the Biostream (1.8 g total protein/l, activity titer 256), was processed for 3 h with 2% (w/v) carrier ampholytes (pH 8–9.5). In this step the last traces of salt and contaminants, which disturb

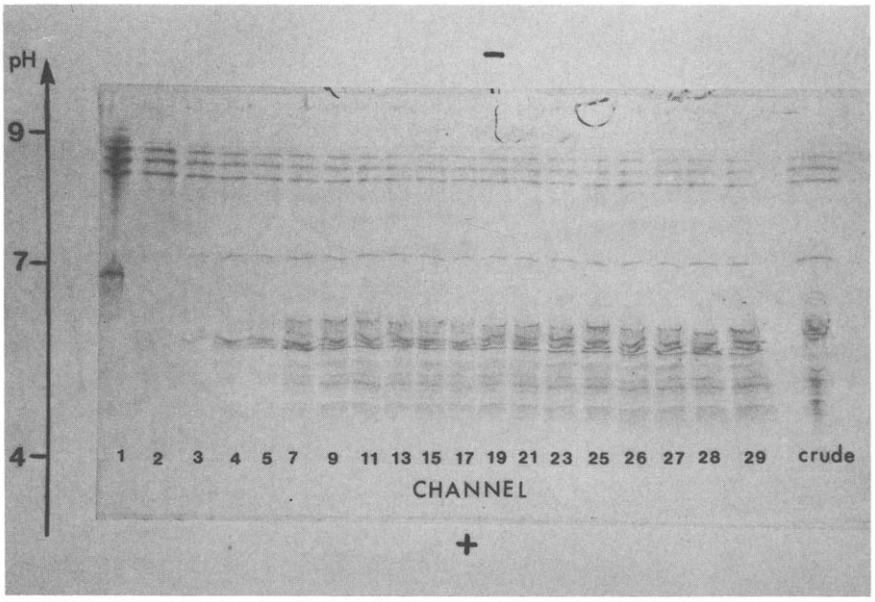

Fig. 10. PAGIEF analysis (stained with Coomassie Blue) showing the distribution of proteins in the 29 Biostream fractions at the beginning of collection. Fraction numbers start at the cathode.

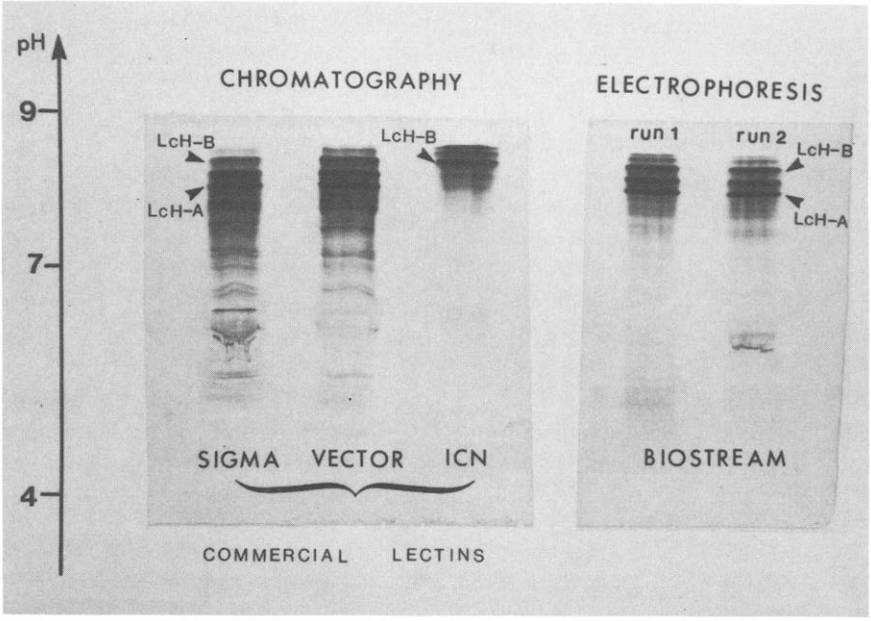

Fig. 11. Silver stained PAGIEF analysis of commercially available lentil lectins from Sigma (No. L-5800, lot 114F-9670), Vector (No. L-1040, lot 50607) and ICN ImmunoBiologicals (No. 79-135-1, lot LB 17-02) in comparison to the Biostream purified product (fraction 1 of two different runs). The ICN product contains LcH-B only, whereas all other samples contain two lectins. Volumes 15 μl of each sample (5 mg/ml) were applied.

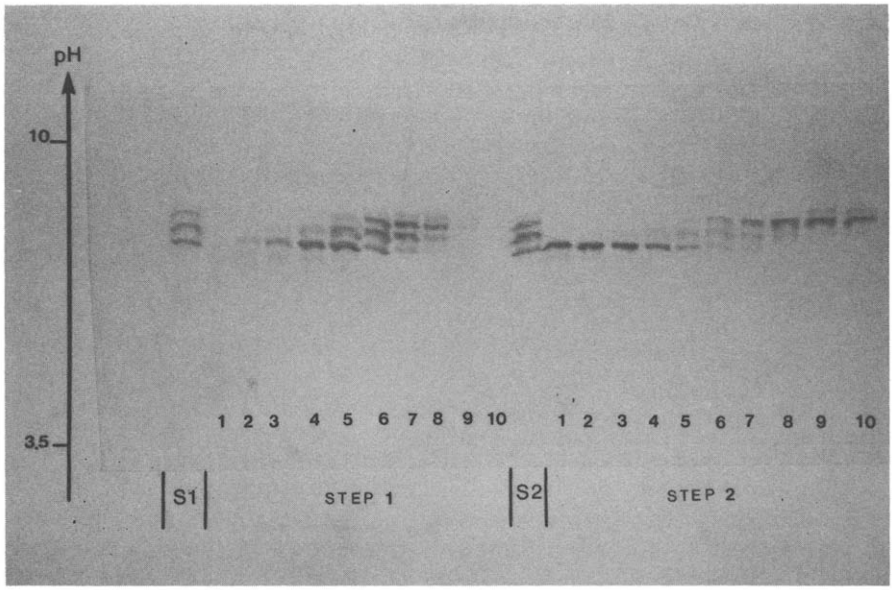

Fig. 12. Distribution of lectins in the ten RIEF compartments for two consecutive runs analyzed by Coomassie Blue stained PAGIEF. For step 1 the inital and final current were 200 and 130 mA respectively and the voltage changed from 340 to 680 V. The corresponding current values for step 2 were 102 and 55 mA at a constant voltage of 700 V.

the linear profile, were removed. Lectins were collected in fractions 5, 6, 7. The pooled fractions had a total protein concentration of 2.4 g/l and an activity titer of 1024 representing a 3 fold enrichment.

(ii) The pooled fractions 5, 6, 7 (72 ml) were diluted to 150 ml and 27 g of urea (3 *M*) were added to dissociate the complex of the two isolectins. This solution, which had a total protein concentration of 1.2 g/l and an activity titer of 512, was focused for 4 h. In this step the two lectins were separated. The highest concentration of LcH-A was recovered from fraction 2 (15 ml): concentration of 1.7 g/l, activity titer of 512. The highest concentration of LcH-B was recovered from fraction 9 (15 ml): concentration of 1.5 g/l, activity titer of 512.

The specific activity is similar in the initial and final solution indicating 3 *M* urea does not deactivate the lectins. In two steps and a total of 7 h 60 mg of each pure isolectin were produced with the RIEF.

CONCLUSION

The present work was intended to compare the use of several electrophoretic instruments for protein purification. Lectins were chosen as a model protein because of their low cost, ready availability and because they have been purified by ion exchange and affinity chromatography. This permits a comparison of electrophoresis to chromatography.

To integrate preparative electrophoretic methods into a purification scheme some important parameters must be considered: (i) Only solutions of low conductiv-

ities can be applied. For biological mixtures, usually, a dialysis or electrodialysis step is a prerequisite. (ii) To obtain the best separation conditions (pH, K) the buffers must be well defined. The choice depends on the type of electrophoretic method and the sample. (iii) In some cases, preliminary purification of the crude extract may be required, especially when the contaminant concentration is high relative to the active compound. In the present work a precipitation was performed before electrophoresis.

Three electrophoretic methods were used successfully to purify lentil lectins. None of the methods were optimized for this specific task, but further optimization is surely possible. Under the condition presented, the Elphor VaP 22 processed 4 mg of lectins/h. However this instrument permits a 10-fold scale-up. The Biostream, designed for large scale processing, usually results in high dilution of the purified sample. The modification of the Biostream described in this paper avoided the dilution and 200 mg/h of lectins were processed in 5–20 l batches of extract.

The RIEF was used as a polishing step to remove the last traces of acidic contaminants and to produce lectins with the highest purity. Lectins, which were prepurified in the Biostream were further enriched 3-fold in the RIEF. In a second RIEF step, the two isolectins were resolved. The small p*I* difference between the two isolectins necessitates the use of a more narrow pH gradient in the second run, which was obtained by pooling the appropriate lectins fractions from the first step. The formation of the complex between the two lectins was avoided by using urea. RIEF favorably competed with affinity chromatography to separate LcH-A and LcH-B.

ACKNOWLEDGEMENTS

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