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LARGE-SCALE PURIFICATION OF PEA GLOBULINS

COMPARISON BETWEEN SIX ANION EXCHANGERS IN MEDIUM-PRESSURE LIQUID CHROMATOGRAPHY

COLETTE LARRÉ* and JACQUES GUEGUEN

Institut National de la Recherche Agronomique, Laboratoire de Biochimie et Technologie des Protéines, rue de la Géraudière, Nantes (France)

SUMMARY

Preparative medium-pressure chromatography of pea globulins was optimized by a comparison of six commercial anion-exchange supports: DEAE-Sepharose CL 6B, DEAE-Sepharose CL 6B Fast Flow, DEAE-TSK 650 M, DEAE-Trisacryl M, DEAE-Spherosil, DEAE-Sephacel. Higher mechanical stabilities and capacities were obtained with DEAE-Sepharose CL 6B, DEAE-Trisacryl M, DEAE-TSK 650 M and DEAE-Sepharose CL 6B Fast Flow, the highest flow-rate (450 ml/h · cm² at 2 bar pressure) being reached on DEAE-TSK 650 M and the highest capacity with both DEAE-Sepharoses (90 mg of pea globulins per ml of gel). Only DEAE-Sepharose CL 6B yielded a total separation of the three major pea globulins (vicilin, convicilin, legumin). However, because of its better mechanical properties, DEAE-TSK 650 M was chosen as the support for the first step in the preparative-scale purification of pea globulins.

INTRODUCTION

The availability of more and more new biologically active molecules has led to the development of large-scale purification techniques. Preparative chromatography, because of its specificity, is well adapted to the production of highly pure and non-denaturated molecules. During the last years, new chromatographic supports, especially ion exchangers, have been developed for high[1–4] as well as for medium-pressure chromatography[5–7]. In this paper, we compare the mechanical stability, capacity and resolution ability of the following anion exchangers: DEAE-Sephacel, DEAE-Sepharose CL 6B, DEAE-Sepharose CL 6B Fast Flow, DEAE-TSK 650 M, DEAE-Trisacryl M and DEAE-Spherosil.

Most of these supports were chosen because of their suitability to medium-pressure conditions. According to the manufacturers, they were developed mainly to support higher flow-rates than those commonly employed in low-pressure chromatography. Because ion-exchange chromatography is increasingly applied in the food

industry[8,9], it seemed of interest to employ these gels with vegetable proteins. Pea proteins were chosen for this comparison first because these proteins have been extensively studied[10–15] and secondly because of their potential as a food[16–18]. Moreover, ion-exchange chromatography had been demonstrated to be a convenient method for separating major pea globulins (legumin, vicilin and convicilin)[14,19]. The physico-chemical characteristics of the proteins explain why DEAE-Sephacel and DEAE-Sepharose CL 6B were included in this comparison, in spite of their lower mechanical stabilities. Both these exchangers are often used for separating legume seed proteins[20–22].

The mechanical properties of the six supports were compared under the same elution conditions; the capacities were not systematically evaluated over pH and ionic strength ranges, but determined only under conditions corresponding to the greater solubility of the proteins. Resolution, on the other hand, was optimized for all exchangers.

EXPERIMENTAL

Chemicals

All biochemicals were obtained from Merck and were of analytical grade.

DEAE-Sepharose CL 6B, DEAE-Sephacel and DEAE-Sepharose CL 6 B Fast Flow were obtained from Pharmacia Fine Chemicals, DEAE-Trisacryl M from IBF Pharmindustry, DEA-Spherosil from Rhone Poulenc and DEAE-TSK 650 (M) from Merck.

Apparatus

The columns K 26/40 and K 100/45 from Pharmacia were used respectively for analytical and large-scale chromatography. Their elution was controlled by a 1130 Ultrograd gradient mixer (LKB) connected to a Frac CC chromatography controller (Pharmacia).

Preparation of proteins

The pea flour was obtained by grinding dehulled seeds (var. Amino) with an industrial roller mill and had an average particle size of about 150 μm.

The crude protein extract was prepared by stirring, for 2 h at room temperature, a slurry of the pea flour in the buffers chosen for eluting the column. The flour/solution ratio was 1 g per 10 ml. According to the solubility properties of pea proteins[23], the buffers used had a pH range of 7–9 and an ionic strength of 0.05 M. The mixture was centrifuged at 3800 g for 30 min and the supernatant introduced on the column after filtration.

Determination of the protein-binding capacity of the gels

The proteins were extracted, as previously described, in 0.05 M Tris–HCl buffer (pH 8), the buffer commonly used for such studies by the manufacturers. The supernatant was obtained after centrifugation at 15 000 g for 15 min. The protein-binding capacity of each gel was determined by the static method, increasing volumes of pea protein extract being added to 3 ml of gel. The resulting slurry was gently mixed on a rotating mixer for 2 h at 25°C and then filtered on a fritted glass. The gel

retained on the filter was washed with buffer until the optical density (280 nm) of the latter decreased to 0. The adsorbed protein fraction was recovered by washing the gel with 0.05 M Tris–HCl pH 8–0.75 M sodium chloride until the optical density of the buffer decreased to zero. The protein concentration of the first filtrate and of the washing buffer was determined by the biuret method yielding respectively the amounts of unadsorbed and fixed proteins. Comparing these values to the protein concentration in the extract enabled the protein recovery to be calculated.

Determination of mechanical stability

The mechanical stability of each gel was established by studying the relationship between the flow-rate and pressure drop. The gel beds packed in a K26/40 column were 30 cm high.

Determination of optimum separation conditions

Packing was carried out under hydrostatic pressure with an aqueous suspension of the supports. The columns were then equilibrated with the buffer used for the experiments at a flow-rate of 40 ml/h · cm². The resolution between pea globulins was optimized for each gel by using a linear gradient of increasing sodium chloride concentration. Two buffer elution systems were tested: phosphate–citrate (pH 7, 0.16 M) and Tris–HCl (pH 8, 0.05 M).

Following these preliminary studies, the optimum resolution conditions for each support were determined and scaled up to preparative assays by using step gradients. The protein composition of each fraction was characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

The resolution, R_S, was calculated by means of the equation

$$R_S = \frac{2 (t_2 - t_1)}{T_1 + T_2}$$

where t_1 and t_2 are the retention times of vicilin and legumin respectively and T the corresponding peak width in min.

The flow-rate and the buffer composition used to elute the proteins are given in the figure legends.

Gel electrophoresis

SDS-PAGE was carried out after reduction of the protein by 2-mercaptoethanol in gradient (10–20%) polyacrylamide slab gels as described by Laemmli[24]. After electrophoresis the gels were fixed with 500 ml of trichloroacetic acid (12.5%, w/w) during 30 min and stained overnight by addition of 50 ml of 0.25% Coomassie G 250. The gel was destained by washing for 2 h with water–ethanol–acetic acid (4.5:4.5:1.5, v/v/v) and then with 5% acetic acid.

RESULTS AND DISCUSSION

Capacity

Pea proteins were extracted from the flour in Tris–HCl 0.05 M, pH 8.0. Fig. 1 shows the increase in the amount of proteins adsorbed with the quantity of proteins
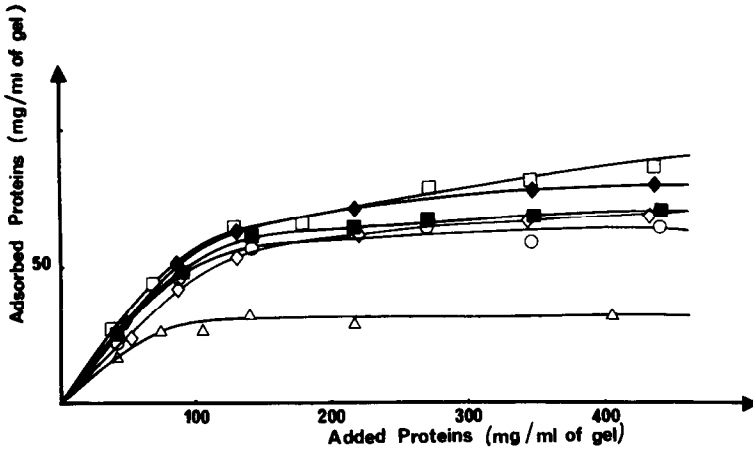
Fig. 1. Capacities of different gels as a function of the amount of pea proteins added. Gels: O—O, DEAE-TSK 650 M; □—□, DEAE-Sepharose CL 6B; ◆—◆, DEAE-Sepharose CL 6B Fast Flow; ■—■, DEAE-Trisacryl; ◇—◇, DEAE-Sephacel; △—△, DEAE-Spherosil.

added to the various supports. According to their protein-binding capacity and the protein recovery, both DEAE-Sepharose CL 6B and DEAE-Sepharose CL 6B Fast Flow had satisfactory performances, the former being the best. DEAE-Sepharose CL 6B fixed 90–100 mg of proteins per ml of gel with a recovery of 97%; DEAE-TSK 650 M, DEAE-Trisacryl and DEAE-Sephacel had lower capacities, between 57 and 70 mg/ml, but showed good recoveries around 90%. DEA-Spherosil gave both a lower binding capacity (25–30 mg/ml of silica) and recovery (75%). The low recovery is certainly related to the nature of the support which is known to exhibit irreversible adsorption phenomena, difficult to suppress even with a good coating[25].

Comparing the support binding capacity for pea proteins to that given by the manufacturers for bovine serum albumin in the same buffer, the main differences are observed for DEAE-TSK 650 M and DEAE-Trisacryl. Whereas the binding capacity for bovine serum albumin was lower in the case of the DEAE-TSK 650 M (26 mg/ml)[26] and higher for DEAE-Trisacryl (100 mg/ml)[27], the binding capacity for pea proteins was almost the same for these gels (60 mg/ml of gel). According to the manufacturer (IBF) of DEAE-Trisacryl, the adsorbing power of this gel decreases with increasing molecular weight of the proteins; in their information note it was shown that the capacity for haemoglobin was lower than that for bovine serum albumin. This phenomenon could explain the lower capacity of DEAE-Trisacryl for pea globulins, the molecular weights for legumin and vicilin being around 360 000 and 180 000 respectively[28]. The better results obtained with TSK in the case of pea globulins compared to bovine serum albumin can be related only to the specific physicochemical properties of these vegetable proteins.

Mechanical properties

DEA-Spherosil has not been included in this comparative study because of the well known rigidity and incompressibility of spherical silica beads[8]. Fig. 2 shows
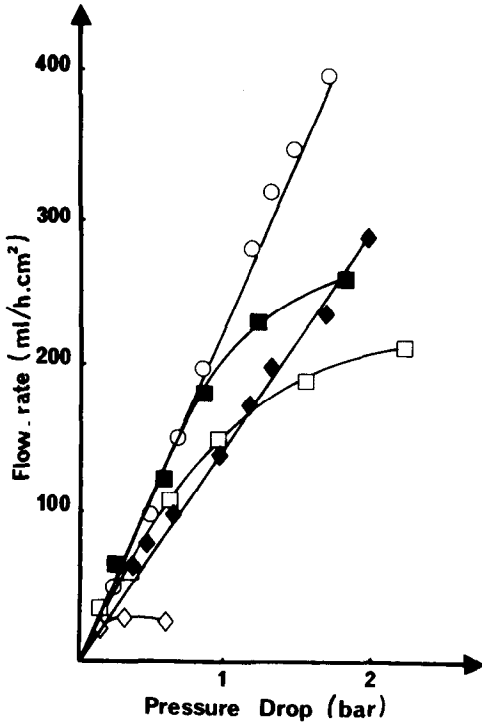
Fig. 2. Flow-rate as a function of the pressure drop on different gel columns (40 cm × 2.6 cm I.D.). Symbols as in Fig. 1.

the relationship between flow-rate and pressure drop. Because of the characteristics of the columns, the pressure at the entrance of the column was not allowed to exceed 2 bar. Up to this pressure, DEAE-TSK 650 M and DEAE-Sepharose CL 6B Fast Flow were not affected; on the other hand, pressures > 1 bar led to compression of DEAE-Trisacryl and DEAE-Sepharose CL 6B. Consequently, at higher pressures the flow-rate no longer increased linearly (Fig. 2). The maximum flow-rate which can be used was 155 ml/h · cm^2 for the DEAE-Trisacryl and 86 ml/h · cm^2 for the DEAE-Sepharose CL 6B. It was only 25 ml/h · cm^2 on DEAE-Sephacel, showing the poor mechanical properties of this gel. Even for DEAE-Trisacryl and DEAE-Sepharose CL 6B the maximum flow-rates were lower than those obtained for DEAE-TSK 650 M (450 ml/h · cm^2) and DEAE-Sepharose CL 6B Fast Flow (280 ml/h · cm^2) at 2 bar pressure.

Fractionation of pea globulins

Since the aim of these experiments was the use of the gels for large-scale chromatography, only the supports which had satisfasctory capacities and mechanical properties were tested for fractionation assays. For these reasons, among the six gels tested, only four were retained: DEAE-Trisacryl, DEAE-TSK 650 M, DEAE-Sepharose CL 6B, DEAE-Sepharose CL 6B Fast Flow. DEA-Spherosil and DEAE-

Sephacel were rejected because of their low adsorption properties and compressibility, respectively.

The studies were carried out by eluting the proteins first by a linear salt gradient and then by steps of increasing salt concentration in order to determine suitable preparative conditions.

Linear gradient conditions. Ion-exchange chromatography conditions were defined on the various gels on the analytical scale by optimizing the resolution between the peaks of vicilin and legumin. It was shown that phosphate–citrate buffers were more efficient for Sepharose gels whereas Tris–HCl buffers were preferable for DEAE-TSK. In both cases, the adsorbed proteins were eluted by a linear gradient of 0 to 0.5 M sodium chloride. At a flow-rate of 40 ml/h · cm^2, resolutions close to 1 were obtained for DEAE-Sepharose CL 6B, DEAE-Sepharose CL 6B Fast Flow and DEAE-TSK.

In the case of DEAE-Trisacryl, the use of a gradient of from 0 to 1 M sodium chloride did not result in desorption of the proteins and simultaneous pH and salt gradients were needed. The pH gradient was established in the range 6–8 by mixing 0.1 M phosphate–citrate and 0.1 M phosphate buffers. Even under these conditions the best resolution between vicilin and legumin was 0.35. Because of this low resolution, DEAE-Trisacryl was not used for separating pea globulins in the following assays.

Step gradient conditions. From these results, extrapolation to step gradients was made for preparative application in order to optimize the elution volume and to
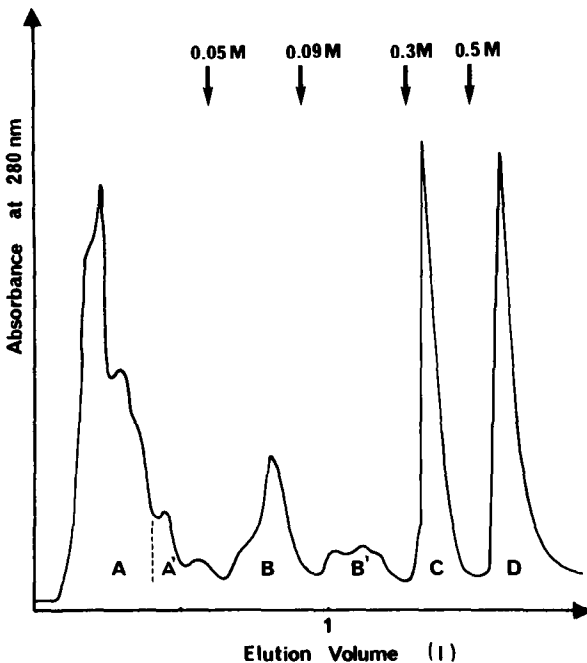

Fig. 3. Ion-exchange chromatography of pea proteins on DEAE-Sepharose CL 6B with step gradients of 0.05, 0.09, 0.3 and 0.5 M sodium chloride in phosphate–citrate buffer (pH 7, 0.1 M). Flow-rate: 40 ml/h · cm^2.

shorten the chromatography. For all experiments the columns (K 26/40) were loaded with 70 ml of crude extract and eluted at a flow-rate of 40 ml/h · cm². On each of the three gels chosen the chromatograms (Figs. 3, 4, 5) exhibited four major peaks. According to electrophoresis, the first peak (A) corresponding to the non-adsorbed proteins was mainly composed of albumins[19]. The second peak (B) contained vicilin, characterized in SDS-PAGE by major bands around MW 50 000, 22 000, 17 000 (Fig. 6). In each case, a band around MW 71 000 could be observed, indicating a contamination of this last fraction by convicilin. Legumin was eluted in the third peak (C), showing in SDS-PAGE strong bands around MW 40 000 and 20 000. The last peak (D) contained non-protein material[19], probably nucleic acids as shown by UV spectroscopy. In order to limit the contamination between vicilin and legumin fractions, an intermediate elution step was applied on both DEAE-Sepharose CL 6B and DEAE-Sepharose CL 6B Fast Flow, leading to the elution of a fraction (B′) containing the three major globulins. The similar results obtained with the two Sepharoses were in accord with previous studies by Berglof and Looney[5]. Those authors observed no difference between these gels except for the flow-rate.

Vicilin and legumin were separated on each of the three gels, but only DEAE-Sepharose CL 6B yielded a vicilin fraction without convicilin when low flow-rates were used. This vicilin fraction was eluted in the shoulder (A′) of the excluded peak
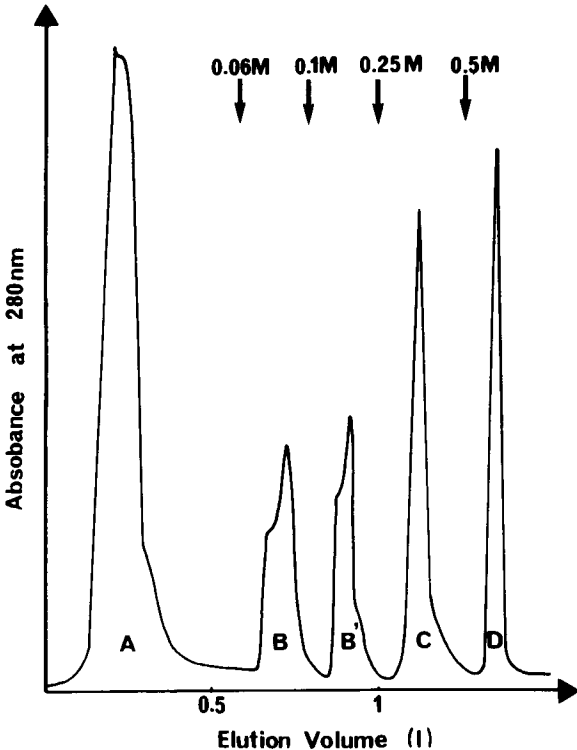

Fig. 4. Ion-exchange chromatography of pea proteins on DEAE-Sepharose CL 6B Fast Flow with step gradients of 0.06, 0.1, 0.25 and 0.5 M sodium chloride in phosphate–citrate buffer (pH 7, 0.1 M). Flow-rate: 40 ml/h · cm².
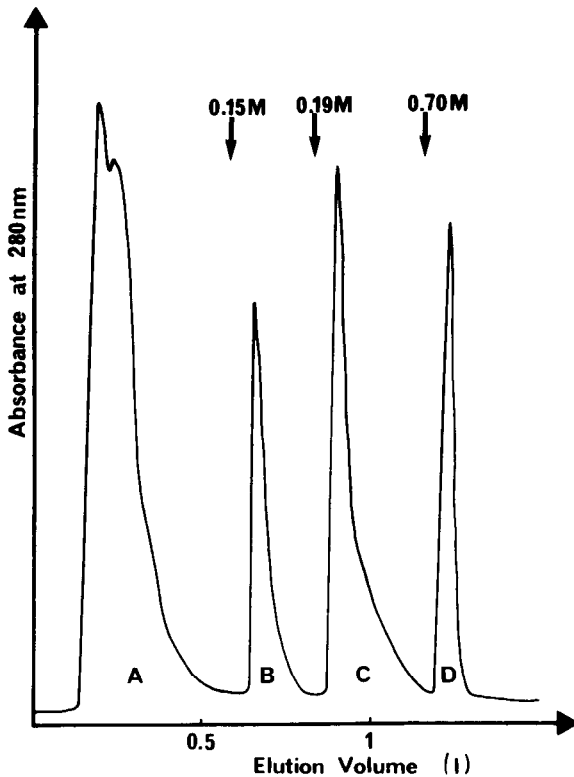
Fig. 5. Ion-exchange chromatography of pea proteins on DEAE-TSK 650 M with step gradients of 0.15, 0.19 and 0.7 M sodium chloride in Tris–HCl buffer (pH 8.2, 0.05 M) containing 0.106 M sodium chloride. Flow-rate: 40 ml/h · cm².

(A). As in the case of DEAE-Sepharose CL 6B, attempts were made to obtain a similar fraction of non-contaminated vicilin on the other gels by increasing the ionic strength of the initial eluting buffer. However, in each case, the excluded fraction of vicilin was contaminated by convicilin. This particular property of DEAE-Sepharose CL 6B could be explained by interactions between the matrix and the convicilin fraction.

CONCLUSION

DEAE-TSK 650 M and DEAE-Sepharose CL 6B Fast Flow characterized by high flow-rate and high capacity could be used in the first step for fractionation of vicilin and legumin. Depending on the objectives, DEAE-TSK 650 M may be preferable because of its better mechanical properties, DEAE-Sepharose CL 6B because of its higher capacity. When separating pea globulins, it was considered that the purified proteins should be recovered as fast as possible in order to avoid microbiological or endogenous enzymatic hydrolysis as well as conformation modifications. For this reason, DEAE-TSK 650 M was chosen for the large-scale assays.
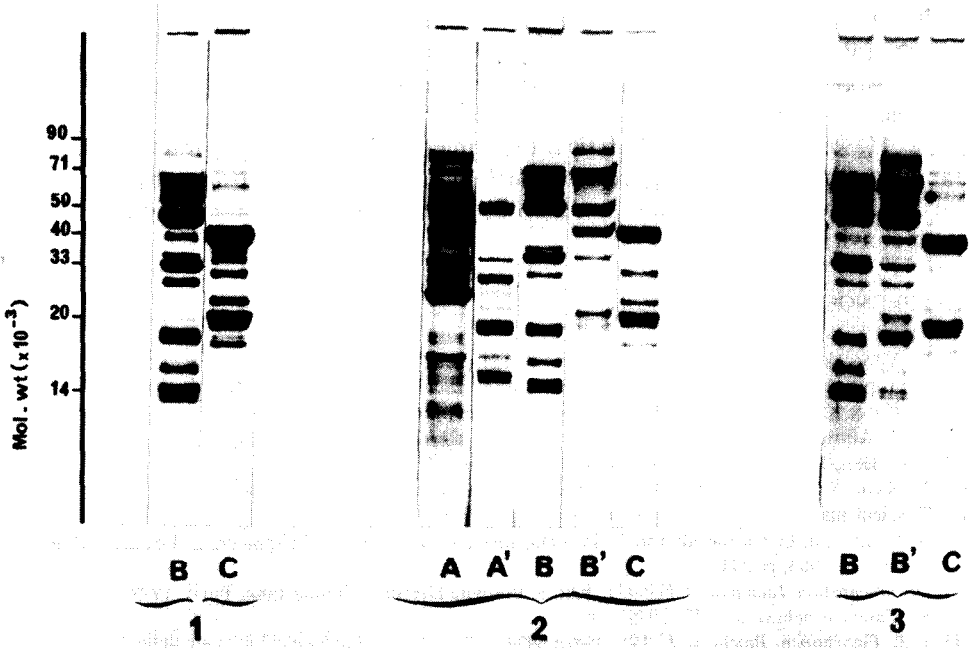
Fig. 6. SDS-PAGE of the chromatographic fractions obtained on the three gels tested: 1, DEAE-TSK; 2, DEAE-Sepharose CL 6B; 3, DEAE-Sepharose CL 6B Fast Flow.
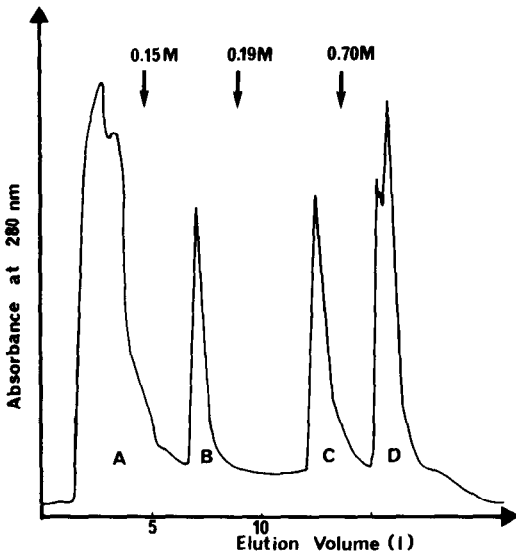

Fig. 7. Preparative ion-exchange chromatography of pea proteins on DEAE-TSK 650 M with step gradients of 0.15, 0.19 and 0.7 M sodium chloride in Tris–HCl buffer (pH 8.2, 0.05 M) containing 0.106 M sodium chloride. Column: Pharmacia K 100/45. Flow-rate: 69 ml/h · cm^2.

With this support, the purification of vicilin and legumin fractions was as good as in the analytical experiments. The elution profile resulting from the preparative chromatography was very similar to the analytical one, as shown by Fig. 7. Moreover, the satisfactory separation between vicilin and legumin was confirmed by electrophoresis.

However, as in the analytical experiments, the vicilin-rich fraction was contaminated by convicilin. In order to purify it, a second chromatography step had to be applied on DEAE-Sepharose CL 6B because of the specific interactions of this gel with convicilin.

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