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Sample displacement mode chromatography: purification of proteins by use of a high-performance anion-exchange column[a]

K. VEERARAGAVAN, A. BERNIER and E. BRAENDLI*

BioSeparation Group, Biochemical Engineering Section, Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec, H4P 2R2 (Canada)

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ABSTRACT

In sample displacement mode (SDM) chromatography, the column is overloaded with sample until it is saturated with the product of interest, while weakly binding impurities are displaced. Because the method makes use of the full capacity of a column, it is interesting for large-scale fine purfication of proteins. By using two columns in series, the product can be separated from both strongly and weakly binding impurities. The working principle of SDM was proved by using Mono Q ion-exchange columns. Crude samples of ovalbumin and soybean trypsin inhibitor were applied to one- and two-column systems and the sequence and purity of both the displaced and the eluted components were determined by off-line analyses. Conditions such as column dimensions, load flow-rate, buffer type and temperature have to be optimized for each target protein and associated impurities in order to maximize the yield and purity by minimizing overlapping.

INTRODUCTION

Displacement mode chromatography has been used to efficiently purify antibiotics [1–3], steroids [3–5], amino acids [6], peptides [3,7,8] and proteins [3,9] at semi-preparative and preparative scales on reversed stationary phases. In this method, the sample mixture in a carrier solvent that has a low affinity for the stationary phase is loaded and the bound components are displaced by a solution of displacer which has a greater affinity for the stationary phase than any of the sample components [10].

This concept has recently been extended to sample displacement mode (SDM) chromatography by Hodges *et al.* [11], who used it to purify peptides with reversed-phase columns. During loading, there is competition among the sample components for the hydrophobic adsorption sites of the stationary phase. The more hydrophobic components compete more successfully for these sites than the more hydrophilic components, which are displaced and eluted from the column. Finally, the adsorbed components are eluted with an aqueous organic eluent. Whereas in ordinary

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displacement chromatography the displacement train fully develops during the elution of the non-saturated column, in the sample displacement mode the bands form only during the load procedure and the columns are fully saturated. Hodges *et al.* [11] used a two-column SDM strategy to purify the peptide of interest from a synthetic mixture of prepurified hydrophobic and hydrophilic impurities. The hydrophobic impurities were adsorbed on the precolumn whereas the hydrophilic impurities were displaced from the main column, leaving the main column filled with the peptide of interest. The adsorbed components from the columns were eluted separately by a solvent gradient. The size of the precolumn was optimized based on the known concentration of the impurities present in the sample.

The purification of peptides by reversed-phase SDM chromatography by Hodges *et al.* [11] is the only report to date. As organic solvents are involved, reversed-phase high-performance liquid chromatographic (HPLC) columns generally cannot be used to separate native proteins. Therefore, in this paper we tried to purify two proteins, ovalbumin and soybean trypsin inhibitor (STI), by SDM chromatography using high-resolution anion-exchange (Mono Q) columns. The matrix of Mono Q columns consists of monodisperse hydrophilic material. The working principle of SDM chromatography and its potential to generate highly purified proteins had to be proved with this column type.

EXPERIMENTAL

Chemicals

Bovine serum albumin (BSA), soybean trypsin inhibitor (STI), trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone treated), Nα-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) hydrochloride and Tris were obtained from Sigma (St. Louis, MO, U.S.A.). Chemicals used for electrophoresis were obtained from Bio-Rad Labs. (Mississauga, Canada). HPLC-grade acetonitrile was obtained from Fisher Scientific (Montreal, Canada). HPLC-grade trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, U.S.A.). All other chemicals were of analytical-reagent grade.

Apparatus

The fast protein liquid chromatographic (FPLC) system consisted of an LCC-500 controller, two P-500 pumps, an MV-7 injection valve, a solvent mixer, a sample loop (50 ml), a UV-1 monitor, a dual-pen recorder and a Frac-100 fraction collector from Pharmacia (Baie D'Urfé, Canada). High-resolution anion-exchange columns, Mono Q 5/5 (Pharmacia), were used in the FPLC unit. In the SDM experiments, the protein samples (200–500 mg dry weight) dissolved in 25 m*M* piperazine–HCl buffer (pH 6.0) (buffer A) were loaded on one Mono Q column or two Mono Q columns connected in series. After loading the samples, the columns were washed individually with buffer A and the adsorbed proteins were eluted with a gradient of sodium chloride in buffer A. The fractions collected were desalted on a PD-10 column (Pharmacia) and used for further analyses. An aliquot (200 μl) of each fraction containing about 0.5 mg of protein was analysed by FPLC on a Mono Q column under the conditions described in Fig. 2, to evaluate its composition.

Another aliquot of each fraction was analysed by HPLC to monitor the composition of the sample. The HPLC analyses were performed with a Waters Assoc.

(Mississauga, Canada) HPLC system consisting of two Model 590 pumps, a Model 490 programmable multi-wavelength detector, a Model 712 WISP autoinjector, a Model 840 data collection system and an LA50 recorder. A Vydac C_{18} reversed-phase column (CSC, Montreal, Canada) was used for this purpose. The proteins adsorbed on the reversed-phase column were eluted by a gradient of 0.1% TFA–acetonitrile in 0.1% TFA–water.

Gel electrophoresis

Electrophoretic analyses were performed in the Bio-Rad system. Selected eluted fractions were analysed for purity by native and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed with a 4% stacking and 10% running gels as described in detail elsewhere [12]. The gels were stained with Coomassie Brilliant Blue R-250 (0.025%) and destained in 10% (v/v) isopropanol containing 10% (v/v) acetic acid as described by Krueger *et al.* [13].

STI assay

STI activity was measured by the method of Fritz *et al.* [14] using BAPNA as substrate. The specific activity of STI was expressed as international inhibitor milli unit (ImU)/mg protein. One ImU is defined as a decrease of $\Delta E = 0.00332$ per min per 3 ml (or 0.01 per 3 min per 3 ml) in the extinction change at 405 nm resulting from the formation of *p*-nitroaniline from BAPNA.

Protein concentration

The protein concentration was determined by the method of Bradford [15] using BSA as standard. STI concentration was determined using a specific absorbance, $A_{1\,cm}^{1\%}$ (280 nm), of 8.9.

RESULTS

Ovalbumin

A typical elution chromatogram of 0.7 mg of ovalbumin sample applied at 1.0 ml/min to a Mono Q column with a capacity of 30–50 mg of protein is shown in Fig. 1. There is one main peak (peak 5) and five minor peaks (peaks 1–4 and 6). When the peak 5 was analysed by SDS-PAGE, it showed a molecular mass of 43 000, indicating that it

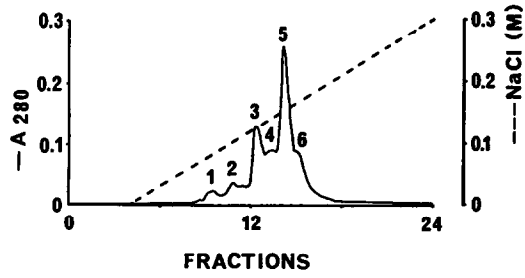

Fig. 1. Typical elution chromatogram of 0.7 mg of crude ovalbumin. Ovalbumin (0.7 mg in 0.5 ml) was loaded on a 5/5 Mono Q column at 1.0 ml/min, washed with 4 ml of 20 m*M* piperazine–HCl buffer (pH 6.0) and eluted with a 0–0.3 *M* linear gradient of NaCl in 20 m*M* piperazine–HCl buffer (pH 6.0) over 20 min.

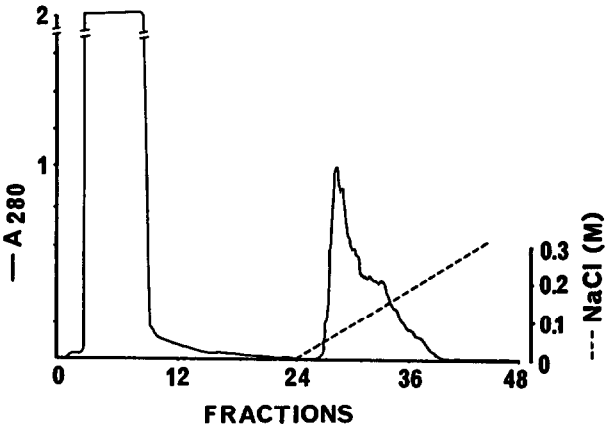

Fig. 2. SDM chromatogram of 200 mg of crude ovalbumin on a 5/5 Mono Q column. The sample (10 mg/ml) was loaded at a flow-rate of 0.2 ml/min, washed with 20 m*M* piperazine–HCl buffer (pH 6.0) at a flow-rate of 1.0 ml/min and eluted at a flow-rate of 1.0 ml/min with a 0–0.3 *M* linear gradient of NaCl in the same buffer for 20 min. Fractions of 2 ml while loading and 1 ml while washing and eluting were collected.

is an ovalbumin peak (data not shown). There is just one major compound (peak 6) in the mixture that binds more strongly than ovalbumin.

To isolate peak 5 from peak 6, which is more anionic, and peaks 1–4, which are less anionic proteins, 200 mg of crude ovalbumin (10 mg/ml) were loaded on one Mono Q column at a flow-rate of 0.2 ml/min (Fig. 2). The column flow-through, wash and eluted fractions were analysed by FPLC with a Mono Q column under standard conditions (Fig. 3).

The analysis of the flow-through fractions showed that after four fractions or 8 ml of sample had been loaded on the column, only proteins of peaks 1–3 were detected in the column effluent. In other words, the column was already saturated with these compounds and they were being displaced by the more strongly binding protein components. Fraction 6 of the flow-through indicated that the product, peak 5, was starting to be displaced and fraction 8 was similar to the load sample; only peak 6 was still fully retained in the column. According to this displacement pattern, we expect the whole amount of peak 6 and part of peak 5 to be bound in the column. The elution profile of the saturated Mono Q column did not allow any interpretation, as distinct protein peaks were not visible. An analysis of the early fraction 28 of the eluted material showed that even after extensive washing of the column with buffer until the absorbance at 280 nm was zero, small amounts of peaks 3 and 4 could still be found in the eluate with the main peak 5 of the product. This finding was unexpected, as peak 5 was already found in the effluent and peaks 3 and 4 should have been displaced. Peak overlapping can obviously be a problem.

To study further the efficiency of displacing the less anionic by the more anionic species in a sample, 500 mg of ovalbumin were passed through two Mono Q columns connected in series. After loading the sample, the columns were separated, washed and eluted individually (Fig. 4). All the flow-through, washing and eluted fractions were analysed not only by FPLC but also by HPLC and native gel electrophoresis.

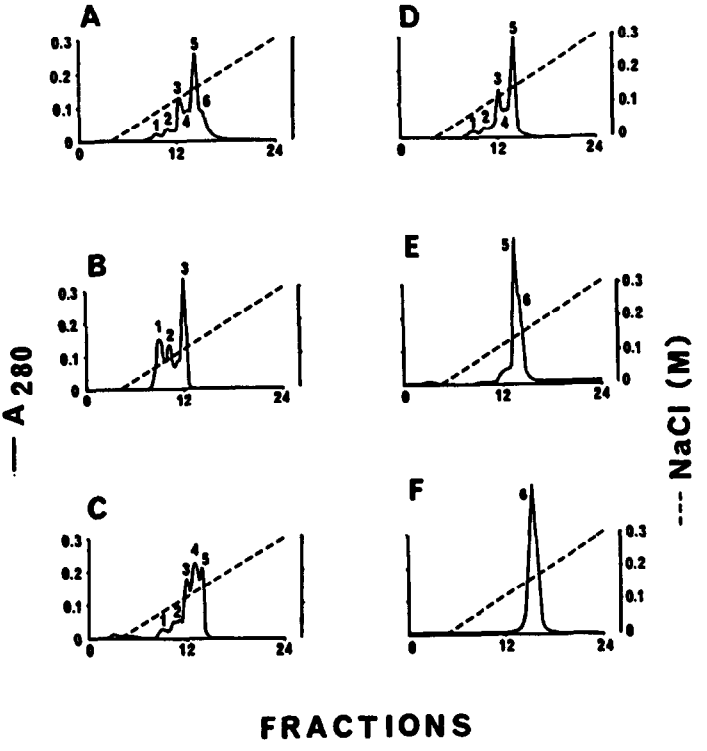

Fig. 3. FPLC analyses of the fractions from Fig. 2. The loading, washing and elution conditions were as in Fig. 1. (A) Crude ovalbumin; (B) fraction 4; (C) fraction 6; (D) fraction 8; (E) fraction 29; (F) fraction 36. Samples of about 0.7 mg of protein were analysed.

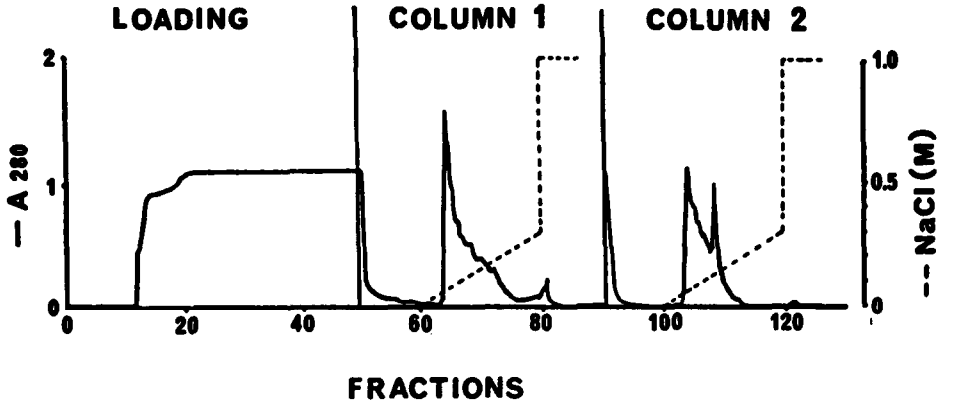

Fig. 4. SDM chromatogram of 500 mg of crude ovalbumin on two 5/5 Mono Q columns connected in series. The sample (10 mg/ml) was loaded at a flow-rate of 0.2 ml/min. After loading, the columns were disconnected and each column was individually washed with 20 m*M* piperazine–HCl buffer (pH 6.0) at a flow-rate of 1.0 ml/min and eluted at a flow-rate of 1.0 ml/min with a 0–0.3 *M* linear gradient of NaCl in the same buffer over 20 min. Fractions of 1 ml were collected.

After the application of about 10–12 ml of protein solution (100–120 mg of total protein) to the column, the absorbance at 280 nm in the effluent started to increase. Different compounds emerged sequentially and could be identified in the collected fractions (Figs. 4 and 5). The ovalbumin could be detected first in fraction 25. At that moment only material of peaks 5 and 6 should theoretically be bound in the two columns. Another 250-mg sample was loaded. The elution of columns 1 and 2 showed that only one wide peak could be detected by FPLC of these fractions (Fig. 5).

The HPLC analyses showed the presence of less anionic proteins which were displaced from both columns in early flow-through fractions (Fig. 6). Similarly, more anionic proteins (peaks 6 and 5) were accumulating in columns 1 and 2, respectively (Fig. 6C and D). However, two peaks were not resolved by the reversed-phase column.

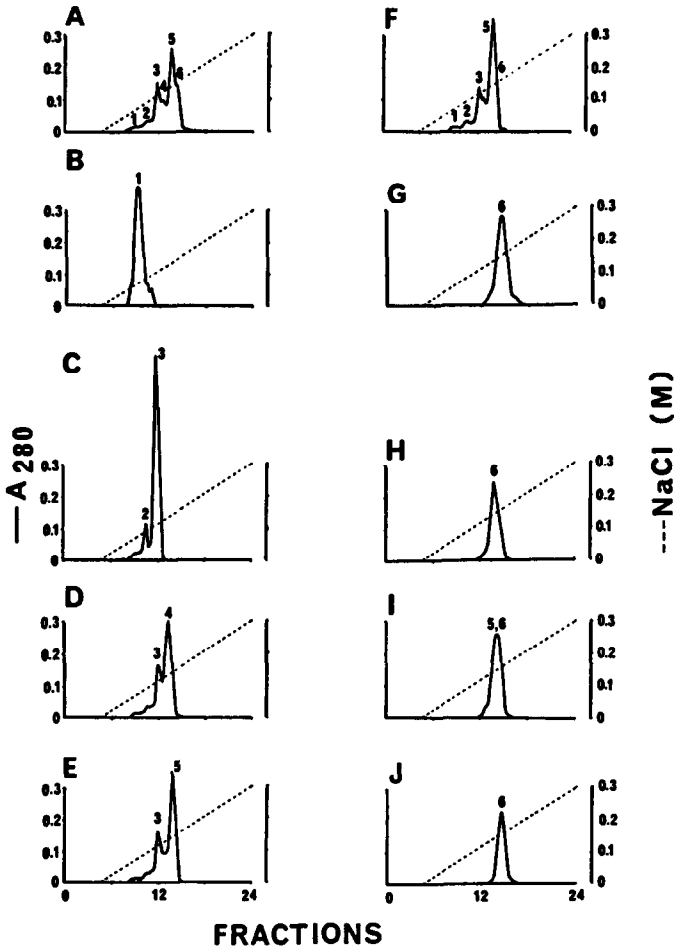

Fig. 5. Analyses of the fractions from Fig. 4. The loading, washing and elution conditions are explained in Fig. 1. (A) Crude ovalbumin; (B) fraction 13; (C) fraction 15; (D) fraction 21; (E) fraction 30; (F) fraction 49 (column I); (G) fraction 66; (H) fraction 70; (I) fraction 105 (column II); (J) fraction 109. Samples of 0.7 mg of protein were analysed.

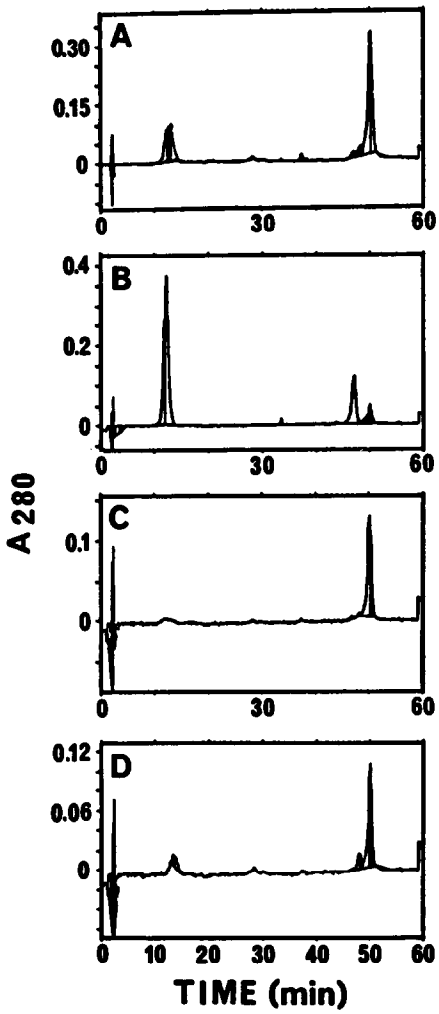

Fig. 6. HPLC analyses of fractions from loading and elution. (A) 250 μg of ovalbumin; (B) fraction 13; (C) fraction 66; (D) fraction 105. Fractions containing about 300 μg of protein were analysed.

Selected fractions were therefore analysed by native gel electrophoresis (Fig. 7), which confirmed the displacement process during the sample loading (Fig. 7C–F). The electrophoretic pattern of the eluate of column 1 (fractions 66 and 70) showed that at least three bands could be distinguished with this method. We expected only two bands, the ovalbumin and the strong binding material of the FPLC peak 6. Only in the elution fractions 109 and 110 of column 2 could highly purified ovalbumin be found.

The working principle having been proved by this first model system, we studied a second sample, which was a crude mixture of a trypsin inhibitor. In addition to analysis by FPLC, HPLC and electrophoresis, the purity and yield of this inhibitor compound could be calculated from activity determination.

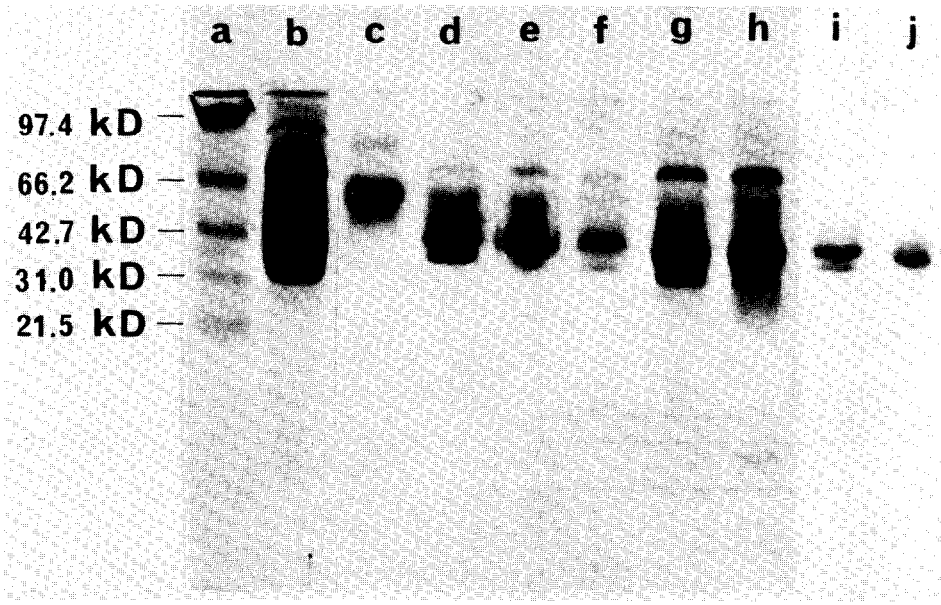

Fig. 7. Native polyacrylamide (10%) gel electrophoresis of loading and elution fractions. (a) Low-molecular-mass standards (Bio-Rad Labs); (b) crude ovalbumin (40 μg); (c) fraction 13 (32 μg); (d) fraction 15 (45 μg); (e) fraction 21 (42 μg); (f) fraction 30 (31 μg); (g) fraction 66 (21 μg); (h) fraction 70 (38 μg); (i) fraction 109 (45 μg); (j) fraction 110 (36 μg). kD = kilodaltons.

Soybean trypsin inhibitor

Fig. 8 is a typical chromatogram of 2 mg of crude STI, showing at least eight distinguishable protein peaks. When these peaks were analysed for trypsin-inhibiting activity, all except peak 6 did not inhibit the enzyme. Therefore, we attempted to isolate the STI peak (peak 6) from the more anionic (peaks 8 and 7) and less anionic (peaks 1–5) proteins. Peak 6 showed a molecular mass of 21 500 by SDS-PAGE (data not shown). Peak 1 consists of material that is not or only slightly retained under the running conditions. Peak 8 only appears at very high salt concentrations compared with the salt gradients used for elution of the compounds.

To purify STI from the crude sample, 500 mg of protein solution (10 mg/ml) was passed at a flow-rate of 0.2 ml/min through two Mono Q columns connected in series. After loading the sample, the columns were washed and eluted individually (Fig. 9). All the flow-through, washing and eluted fractions were analysed by FPLC, HPLC and native gel electrophoresis.

The absorbance at 280 nm of the effluent started to rise after 22 fractions or 27 ml had been collected. The beginning (Fig. 10B), middle (Fig. 10C) and end (Fig. 10D) of the flow-through fractions analysed by FPLC are shown. The less anionic proteins were displaced one by one from both columns. The product peak 6 started to be displaced and could be detected by FPLC in fraction 30. The more strongly binding component of peak 8 could not be found in the column effluent during loading and was not found in the elution fractions of column 2. Even after extensive washing, the

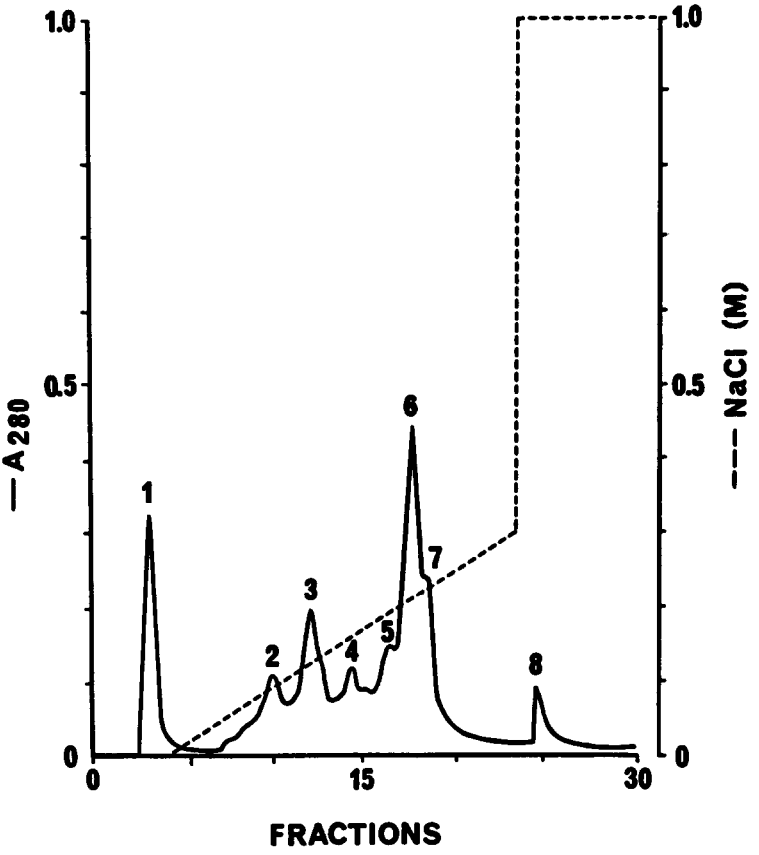

Fig. 8. Typical elution chromatogram for 2 mg of crude STI. STI (2 mg in 0.5 ml) was loaded on a 5/5 Mono Q column at a flow-rate of 1.0 ml/min, washed until 4.0 min with 20 m*M* piperazine–HCl buffer (pH 6.0) and eluted with a 0–0.3 *M* linear gradient of NaCl in the same buffer for 20 min and then with 1.0 *M* NaCl for 10 min.

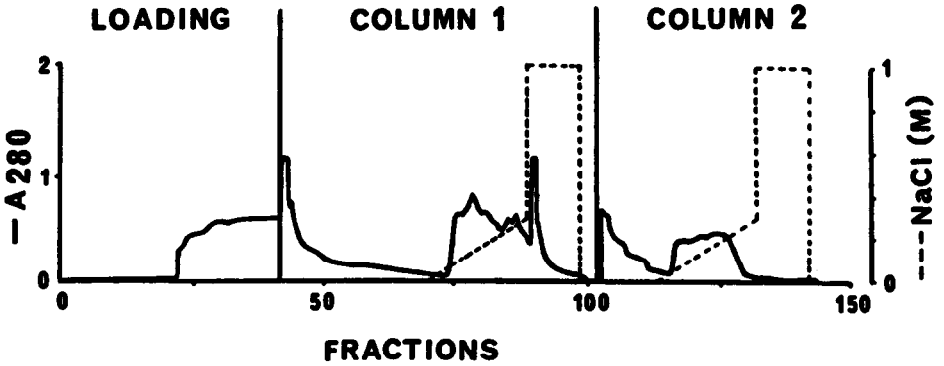

Fig. 9. Chromatogram of 500 mg of crude STI on two 5/5 Mono Q columns connected in series. The sample (10 mg/ml) was loaded at a flow-rate of 0.2 ml/min. After loading, the columns were disconnected and each column was washed individually with 20 m*M* piperazine–HCl buffer (pH 6.0) at a flow-rate of 1.0 ml/min and eluted at a flow-rate of 1.0 ml/min with a 0–0.3 *M* linear gradient of NaCl in the same buffer for 20 min and then with 1.0 *M* NaCl for 10 min. Fractions of 1.2 ml while loading and 1.0 ml while washing and eluting were collected.

TABLE I

PURIFICATION OF SOYBEAN TRYPSIN INHIBITOR BY SAMPLE DISPLACEMENT MODE CHROMATOGRAPHY

Fraction	Total protein (mg)	Specific activity (ImU/mg protein)	Purification (fold)	Recovery (%)
Crude	500	1940	1.0	100
Column 1	26	3233	1.7	8.7
Column 2	22	3798	2.0	8.8

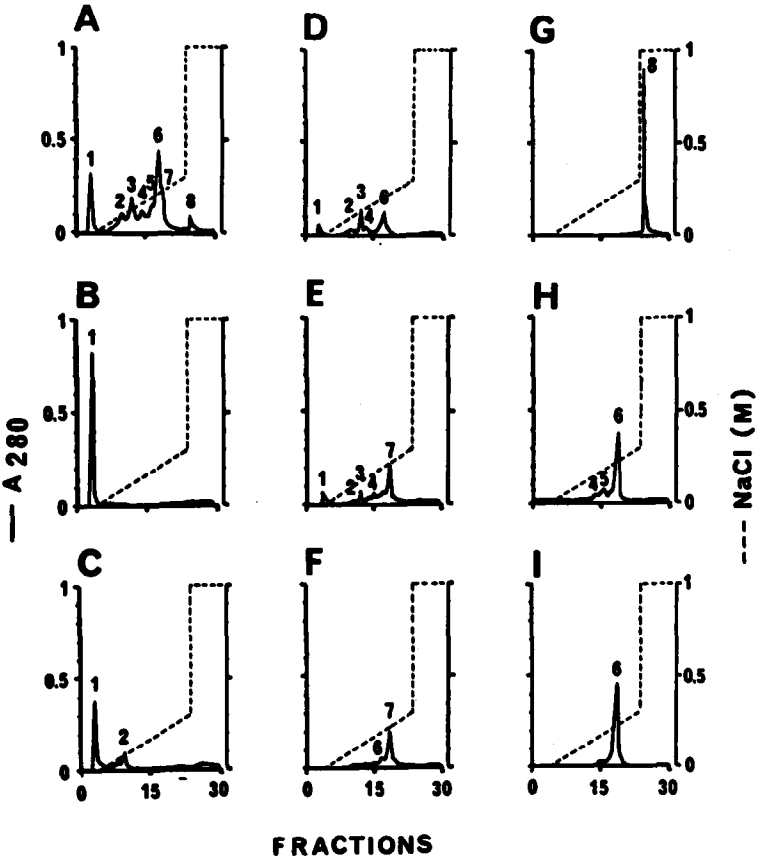

Fig. 10. Analyses of fractions (Fig. 9) by elution chromatography. The loading, washing and elution conditions were as in Fig. 8. (A) Elution chromatogram of 2 mg of crude STI; (B) fraction 5; (C) fraction 25; (D) fraction 30; (E) fraction 40; (F) fraction 77; (G) fraction 90; (H) fraction 122; (I) fraction 125. About 0.5 mg of protein was analysed for each fraction.

absorbance at 280 nm indicated that proteins were leaching from the column. The analysis of the wash fractions of columns 1 and 2 showed that material of peaks 2–6 was flushed from the columns. The wash buffer obviously not only removed the protein material in the void volume of the columns, but also displaced the less tightly binding components.

The first fractions eluted from column 1 contained product peak 6 and minor peaks 3–5. Already fraction 77 showed detectable amounts of impurity peak 7. Peak

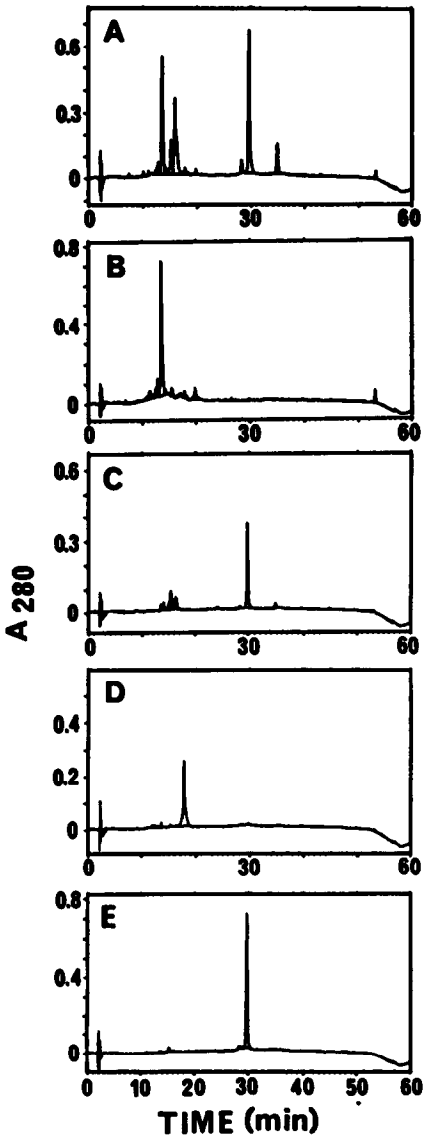

Fig. 11. HPLC analyses of the loading and eluted fractions. (A) 100 μg of crude STI; (B) fraction 5; (C) fraction 78; (D) fraction 90; (E) fraction 127. Fractions containing about 70 μg of protein were analysed.

7 is found mainly in fractions 78–87, simultaneously with peak 6. Only after increasing the salt concentration could the accumulated peak 8 be eluted as a highly purified fraction from column 1.

Column 2 was expected to be saturated by less strongly binding compounds, and this was confirmed by the FPLC analysis of the fractions. The main peak was STI, accompanied by peaks 3–5 in the early fractions. Peaks 7 and 8 could not be detected in these highly pure samples. Active fractions (123–126) were pooled and were found to be more than 95% pure by FPLC; they had a specific activity of 3798 ImU/mg protein (Table I). A two-fold purification of STI was achieved by this method. Nearly homogeneous protein was recovered from the second column. However, the STI recovered from the pooled fractions (77–80) from the first column had a specific activity of 3233 ImU/mg protein (Table I). The STI obtained from the selected fractions of columns I and II amounted to 26.19 and 22.41 mg of STI activity, accounting for 8.7 and 8.8% recoveries, respectively.

HPLC of the fractions revealed that the STI obtained from the later fractions in the second column (Fig. 11E) was homogeneous. However, STI recovered in the earlier eluting fractions from the first column contained a few less anionic impurities (Fig. 11C). These findings demonstrate that peaks 7 and 8 are efficiently adsorbed by the first column, but it is difficult to displace totally the less strongly binding compounds.

The fractions were analysed by native gel electrophoresis (Fig. 12). The results (Fig. 12c–e) confirmed that the different components in the mixture (Fig. 12b) were well displaced during sample loading starting with peak 1. The product peak 6 showed up in the latest load fractions. The two columns were therefore saturated with the components of peaks 6, 7 and 8. Fig. 12f and g show that highly pure material of peak 8 is eluted from column 1 under isocratic conditions with 1 *M* sodium chloride in the elution buffer. Electrophoretically pure STI could be eluted from column 2, as shown in Fig. 12h–j. As the concentration of STI in the eluate was very high, a large amount of material could be loaded directly on the gel.

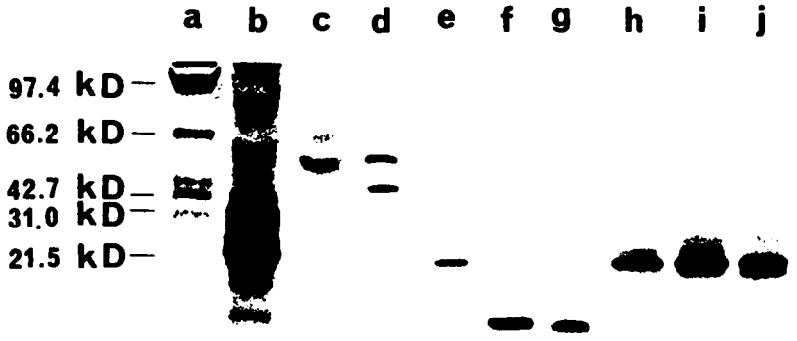

Fig. 12. Native polyacrylamide (12%) gel electrophoresis of the loading and eluted fractions. (a) Low-molecular-mass standards (Bio-Rad Labs.); (b) crude STI (40 μg); (c) fraction 5 (28 μg); (d) fraction 15 (24 μg); (e) fraction 40 (24 μg); (f) fraction 89 (23 μg); (g) fraction 90 (26 μg); (h) fraction 122 (29 μg); (i) fraction 125 (32 μg); (j) fraction 127 (26 μg). kD = kilodaltons.

DISCUSSION

By ordinary linear gradient elution chromatography on Mono Q, the number of components in the two samples ovalbumin and STI, and their relative binding strengths could be determined according to their elution sequence. Even by loading less than 3% of the column capacity in both examples, no baseline resolution was possible. In the overload run, the load flow-rate was reduced to 20%. By analyses of the effluent fractions in one-column experiments, we observed the expected competition between the various proteins present in the mixtures for the limited number of binding sites available in the column, which resulted in a sample displacement process. The elution pattern based on absorbance at 280 nm did not allow any interpretation concerning fraction composition. The analyses had to be done by high-resolution methods.

The results of the trials with ovalbumin gave valuable hints concerning the strategy of purification by sample displacement chromatography. Two situations are very favourable: (a) the product peak is the first peak in the elution chromatogram; in this instance, pure product can be collected in the column effluent until the column becomes saturated with the next most strongly binding compound. This application is only of interest when the product is a major component of the mixture, *i.e.*, the column has a reasonable capacity for the impurities. (b) The product is the most strongly binding compound in the mixture; in this instance, it can displace major amounts of impurities and then be eluted from a one-column system. However, these two favourable situations will rarely be met.

The next step towards a multi-column system, in which theoretically every component of the protein sample could be fixed to a column of the proper dimensions, was a two-column arrangement. The results indicated that the impurities that bind more strongly than the products could be caught in the first column. We expected the second column to be saturated with pure product only. The early eluted fractions in both examples, however, contained traces of weaker binding impurities, indicating that band overlapping due to semi-quantitative displacement will be a challenge in process development. We had not optimized the size of the columns to capture impurities and products and therefore were only able to collect a small percentage of the applied amount as pure products. To evaluate the required dimensions of a two-column system the breakthrough curves of the product and the next more strongly binding impurity will have to be determined. As the two-column approach in SDM leads to columns fully saturated with only the product of interest, the method enables the user to make maximum use of the binding capacity of the column.

These trials were planned to test the feasibility of SDM ion-exchange chromatography. The results show the need for systematic parameter variation in order to optimize throughput, yields and purity. Process optimization and the necessary automation require on-line analysis of the column effluent fractions.

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