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Preparative anion-exchange chromatography of soybean trypsin inhibitor: the alternative of column-overload methods

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ABSTRACT

The objective of preparative separation is to purify the largest amount of material in the shortest time and at a minimum cost, *i.e.* to maximize throughput. One of the techniques for increasing throughput is to overload the column while maintaining purity and cycle time at the same level. This principle is applied in sample displacement mode chromatography, in which the column is overloaded with sample mixture until it is completely saturated. Soybean trypsin inhibitor was purified from a crude protein extract by this technique using an analytical anion-exchange column with small particle size (20 μm). The comparison of these results, using the criterion of throughput, with those derived from a conventional scale-up, using a 40-μm preparative column, led to the conclusion that the overloaded 20-μm column gave a higher throughput than the 40-μm column.

INTRODUCTION

The study of the inhibition of proteases is a major tool in the identification of active-site residues of these enzymes and in the analysis of the structure and function of the active centre. Inhibition reactions are also important for reasons connected with the need to stop proteolytic reactions, for the purpose of isolating native intact proteins from biological tissues. Low-molecular-mass inhibitors of proteolytic enzymes are widely distributed among plant species and tend to accumulate in storage structures, such as tubers, fruits and seeds. The seeds of leguminous plants are particularly rich sources of protease inhibitors. Soybean seeds are known to contain two main classes of protease inhibitors: the Kunitz-type inhibitors and the Bowman–Birk inhibitors. Both types inactivate serine proteases (trypsin, chymotrypsin and elastase).

The Kunitz-type inhibitors are globular proteins of *ca.* 20 000–25 000 molecular mass with few disulphide bonds, and are specific mainly for trypsin. Soybeans also contain a number of closely related isoinhibitors of lower molecular mass (6 000–10 000), widely known as Bowman–Birk inhibitors. These are highly crosslinked with disulphide bonds and are doubled headed, *i.e.* they have independent binding sites for trypsin and chymotrypsin.

This paper describes the isolation of soybean trypsin inhibitor (STI) from soybean meal. The soybean meal was first defatted by extraction with petroleum spirit. The defatted and dried meal was then extracted with Tris–HCl buffer. The supernatant recovered after decantation and filtration was clarified by centrifugation and filtration. A lot of protein contaminants were then removed by acidic precipitation. After concentration by ultrafiltration, the sample was applied to

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an anion-exchange column. This final step is of great importance in the process and its optimization might significantly improve the economics of the whole process. Experimental data concerning anion-exchange chromatography of a synthetic mixture of STI was presented by Tice *et al.* [1]. It was shown that overloading the column was a good way to improve the throughput, which is one of the main parameters in preparative chromatography. It is defined as mass of sample purified/unit of time/unit of bed volume (mg/min/ml).

In this work a similar approach was chosen but was applied to a processed crude extract, *i.e.* products obtained at the end of the purification described above. An analytical separation performed on a 20-μm column enabled operating conditions for the preparative chromatographic separation to be defined. The final step of purification, *i.e.* the preparative anion-exchange chromatography, was performed in two ways:

(1) On a large-scale system, with a preparative 40-μm column. Operating conditions were extrapolated from the analytical separation on the 20-μm column by conventional scale-up. The 20-μm and 40-μm columns were loaded until the same fraction (*ca.* 10%) of the total capacity was reached.

(2) On the 20-μm column operated in conditions of extreme overloading. In this case, the column was loaded until the entire volume of the column was saturated.

The two separations were then compared by using the criterion of throughput as defined above.

EXPERIMENTAL

Materials

Soybean meal used was from variety Cador (Midi-Pyrenées, France) and after harvesting was stored at *ca.* 10°C. Trypsin from porcine pancreas (type IX), BAPA (N-benzoyl-DL-arginine *p*-nitroanilide), PMSF (phenylmethylsulphonyl fluoride) and trypsin inhibitor (Kunitz type I-S) were obtained from Sigma (St. Louis, MO, USA). Columns and gels were from Sepracor, Bioprocessing Division-IBF (Villeneuve la Garenne, France). The 20-μm column was a D-Zephyr/10 column of 9 cm bed height, 1 cm I.D. and 7 ml bed volume. The adsorption capacity for standard molecules was 110 mg/ml (800 mg) bovine serum albumin (BSA) in 0.05 *M* Tris–HCl buffer (pH 8.6). The 40-μm column was a VPC 15 × 1250 filled with DEAE-Spherodex M. The column had a bed height of 9.04 cm, an I.D. of 1.5 cm and a bed volume of 16 ml. The adsorption capacity for standard molecules was 90 mg/ml (1440 mg) BSA in 0.05 *M* Tris–HCl buffer (pH 8.6). Both ion exchangers had an average porous diameter of 0.1 μm and were rigid microbeads consisting of a network of silica and ionizable dextran, which totally covered the silica. In both gels, the dextran polymer carried the classical cationic goups DEAE (diethylaminoethyl).

Purification methods

A 100-g sample of soybean meal was defatted with 1 l of petroleum spirit by stirring (in a 3-l Erlenmeyer flask) for 4 h at 4°C, according to the method of Hu and Esen [2]. The meal was recovered by paper filtration in a Büchner filter, and dried by evaporating the petroleum spirit at room temperature. The defatted meal was then extracted by stirring for 2 h at 4°C with 1 l of 50 m*M* Tris–HCl (pH 8.2) containing 0.3 m*M* PMSF, to avoid the degradation of the STI by proteases present in solution [3]. The solid phase was discarded by filtration on stainless-steel sieves. The liquid phase was centrifuged at 5000 *g* for 5 min and the pellet was eliminated. This operation was repeated twice. The supernatant was then filtered through two types of cellulose acetate filter (pore sizes 1.2 μm and 0.65 μm) placed in a plastic inline filter holder. Finally it was filtered with a hollow-fibre ultrafiltration device containing cellulose membranes with 0.2 μm pore size. The sample was adjusted to pH 5.0 with 0.5 *M* HCl. Precipitated proteins were removed by centrifugation at 5000 *g* for 5 min and then discarded. Salts contained in the sample were eliminated by diafiltration by addition of 10 volumes of 20 m*M* Tris–HCl (pH 8), using a hollow-fibre ultrafiltration device with a membrane of 10 000

molecular mass cut-off. After this operation, the sample was concentrated by a factor of 10, by ultrafiltration at 3.5 bar. The proteins were then purified by anion-exchange chromatography using an FPLC system (Pharmacia). They were detected by measurement of their absorbance at 280 nm, and 5-ml fractions were collected and analysed for protein content and antitryptic activity.

Total protein assay and antitryptic activity measurements

The total protein concentration was assayed by the method of Bradford using the Bio-Rad dye reagent. Pure STI (Kunitz type I-S) from Sigma was used as a standard to calibrate the assay.

The inhibitor activity was assayed according to the method of Liu and Markakis [3]. Calibration was carried out with pure STI (Kunitz type I-S) from Sigma, by incubating various amounts of inhibitors with a constant amount of a trypsin solution and measuring the residual enzyme activity. This assay was most reliable in the inhibition range 30–70%, which achieved a linear plot for amount of inhibitor against percent inhibition. The assay led to constant over-estimation of the mass of trypsin inhibitor, owing to impurities (especially salts) present in the product used for calibration. For this reason, results obtained by this assay were expressed in mg^{equ} (mg equivalent as related to the STI specific activity).

Computation of throughput and yield

Fractions with a specific activity (antitryptic activity (mg^{equ})/total protein (mg)) higher than 25 mg^{equ}/mg were collected, and the throughput obtained with these mixed fractions was calculated as follows: STI in collected fractions (mg^{equ})/cycle time (min) × bed volume (ml).

The yield was defined by: STI in collected fraction (mg^{equ})/STI in sample loaded (mg^{equ}).

Scale-up to preparative chromatography

To scale-up analytical separation to preparative while retaining the same resolution, it is necessary to have $N_1 = N_2$, which leads, in case of mass transfer limited separation efficiency [4], to $L_1/u_1 d_{p1}^2 = L_2/u_2 d_{p2}^2$ (N is the number of theoretical plates, L the column length, u the linear velocity and d_p the particle diameter). This scaling-up maintains the same resolution but usually column manufacturers admit a loss of resolution and their scaling-up leads to higher operating velocities.

RESULTS

First steps of the purification

After each step of purification, the total proteins in solution and the antitryptic activity were assayed, providing the yields and purification factors given in Table I for the different steps. In

TABLE I

PURIFICATION SCHEME, YIELDS AND PURIFICATION FACTORS OF THE DIFFERENT STEPS

Purification step	Specific activity (mg^{equ}/mg)	Yield	Purification factor
Soybean meal			
Defatting with 1 l petroleum spirit, 4 h, 4°C and drying			
Tris–HCl extraction 2 h, 4°C, with 50 m*M* Tris–HCl buffer (pH 8.2)	0.38		
Centrifugation (5000 *g*, 5 min) repeated twice	0.42	0.99	1.1
Supernatant dilution and filtration with cellulose acetate filters (pore sizes 1.2 and 0.65 μm)	0.43	0.77	1.02
Ultrafiltration 0.2 μm	0.83	0.95	1.93
Acidic precipitation by adjustment to pH 5 with 0.5 *M* HCl	4.5	0.95	5.42
Ultrafiltration (diafiltration and concentration) cut-off 10 000	5.0	0.42	1.1

our conditions, according to Liu and Markakis [3], the impurities present in our samples did not affect the validity of trypsin inhibitor activity measurements. Consequently, these measurements gave the amount of really active protein recovered after the purification, which was in our opinion the most important parameter. After removal of lipids, 92 g of soybean meal were obtained and *ca.* 35 g of total protein were extracted with Tris–HCl buffer. The extract was then clarified by centrifugation and filtration. After acidic precipitation, only 2 g of total protein were recovered in the supernatant, including most of the STI. The purity was thus strongly enhanced during this step. After diafiltration and concentration, the sample was used for anion-exchange chromatography.

Anion-exchange chromatography

Analytical anion-exchange chromatography. An analytical chromatogram of the crude STI sample on the 20-μm column is shown in Fig. 1. The elution conditions (pH and gradient slope) were optimized in order to obtain a correct resolution of the sample components. This optimization enabled operating conditions for the two preparative anion-exchange chromatographies to be defined.

Preparative anion-exchange chromatography with the 40-μm column. This chromatography involved the use of a large column under non-overloaded conditions, *i.e.* operated in elution mode. The elution conditions (pH, gradient slope and sample loading) were extrapolated from the analytical values. The linear velocity of the mobile phase was set at 0.96 cm/min (volumetric flow-rate 1.7 ml/min), corresponding to the scale-up formula (see Experimental). Although this condition led to the same high resolution as the analytical separation, the increase in the mass of sample purified (630 mg^{equ} of STI) did not compensate for the increased cycle time (285 min) and the throughput obtained was very low (0.14 mg^{equ}/min/ml). Therefore a linear velocity three times higher than predicted by the formula was used, which corresponded to the flow-rates recommended by the manufacturer. The chromatogram obtained is shown in Fig. 2. The elution profile parallels the analytical separation (Fig. 1), with a moderate loss of resolution. The total time for sample loading and gradient elution (cycle time) was 100 min.

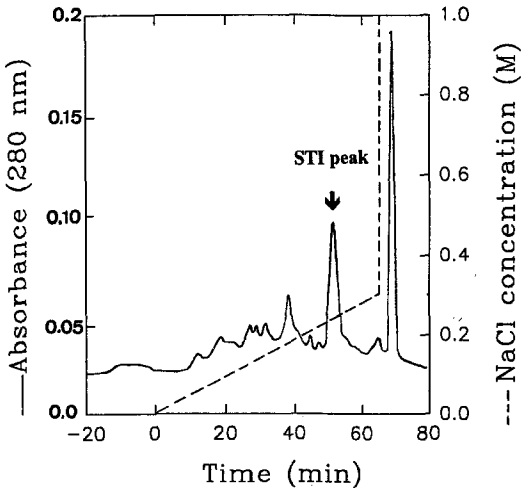

Fig. 1. Analytical chromatography on the 20-μm column. Operating conditions: buffer A, 20 m*M* Tris–HCl (pH 8); buffer B, A containing 1 *M* NaCl; gradient, 0 to 30% of buffer B; sample load, 70 mg of protein; flow-rate, 3.0 ml/min; gradient volume, 171 ml. The peak for STI is indicated.

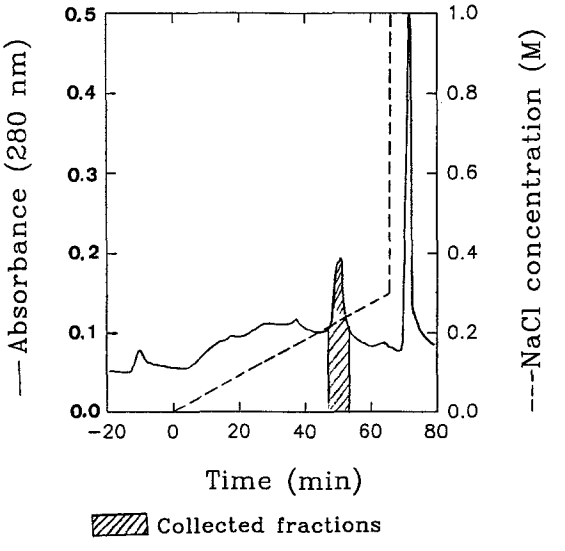

Fig. 2. Preparative chromatography on the 40-μm column. Operating conditions: buffer A, 20 m*M* Tris–HCl (pH 8); buffer B, A containing 1 *M* NaCl; gradient, 0 to 30% of buffer B; sample load, 140 mg of protein; flow-rate, 5.1 ml/min; gradient volume, 391 ml.

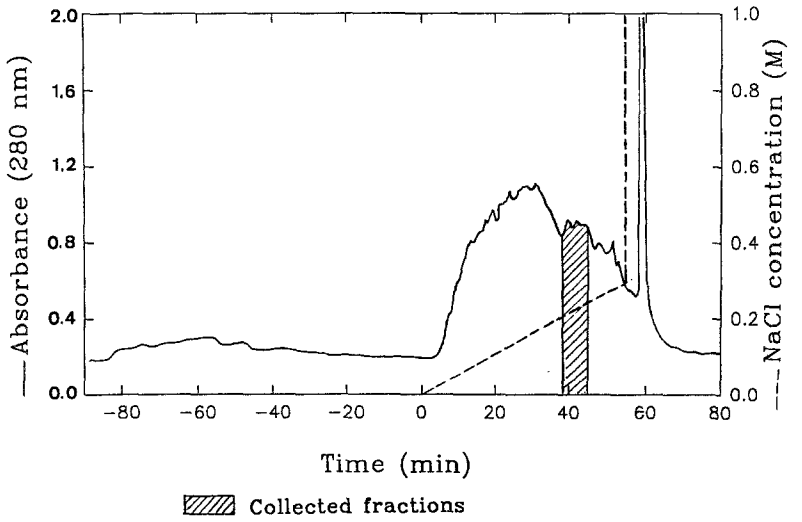

Fig. 3. Preparative chromatography on the 20-μm column. Operating conditions: buffer A, 20 m*M* Tris–HCl (pH 8); buffer B, A containing 1 *M* NaCl; gradient, 0 to 30% of buffer B; sample load, 700 mg of protein; flow-rate, 3.0 ml/min; gradient volume, 171 ml.

Preparative anion-exchange chromatography with the 20-μm column. In this preparative run, the column was saturated with sample components and eluted with an NaCl gradient, *i.e.* the column was operated in displacement mode. The elution conditions (pH, gradient slope, linear velocity) were the same as for analytical separation. The crude STI sample was pumped into the column in 100 min. The column was then eluted at 3.0 ml/min in 80 min and the total cycle time, including 10 min for recycling the column, was 190 min. The chromatogram obtained is shown in Fig. 3. Although no resolved peaks appear on this chromatogram, the analysis of collected fractions revealed that the product of interest was localized in a zone with a relatively high purity.

Throughput evaluation. In both preparative anion-exchange chromatographies, fractions with a specific activity higher than 25 mg^{equ}/mg were isolated and the throughputs were calculated. These results correspond to the throughput of active molecule, with a purity superior to a given limit. Results are presented in Table II. The overloaded 20-μm column achieved the better throughput. The yield of product with a purity greater than 25 mg^{equ}/mg was low, but it might easily be enhanced by recycling the feed.

TABLE II

CALCULATION OF THE THROUGHPUTS OBTAINED WITH THE TWO DIFFERENT SYSTEMS OF PREPARATIVE ANION-EXCHANGE CHROMATOGRAPHY

	Non-overloaded 40 μm-column	Overloaded 20 μm-column
Loaded sample (mg of protein)	140	700
STI in loaded sample (mg^{equ})	700	3500
STI in collected fractions (mg^{equ})	490	910
Cycle time (min)	100	190
Specific activity of collected fractions (mg^{equ}/mg)	28	27
Yield of STI (%)	70	26
Throughput ($mg^{equ}/min/ml$)	0.31	0.68

As a further check on the purity of the products, we analysed the collected fractions by anion-exchange chromatography (using the analytical method on the 20-μm column) and compared them with the high-grade type I-S STI from Sigma analysed under the same conditions. The data indicated that the isolated STI fractions were comparable with the commercial product in chromatography on both the 20- and the 40-μm columns.

CONCLUSION

Among all the techniques devoted to purification of proteins, ion-exchange liquid chromatography is one of the most efficient. These results show that scaling-up an operation by empirical extrapolation of analytical separation was far from being the best choice to develop this technique to a preparative scale, where the concept of throughput is of major importance. Indeed, in this work, the overloaded 20-μm column achieved the better throughput and the drawback of a low yield might easily be overcome by using a recycled feed. This good throughput was mainly due to a larger use of the stationary phase volume, which was close to saturation. However, in this case, the separation was driven not only by the specific affinity of the solutes for the stationary phase but also, to a larger extent, by the competition of different solutes for the adsorption sites. This competition led to displacement effects that forced constituents to organize themselves spatially under the form of apparently non-resolved chromatograms, in which concentrated and separated bands of components might nevertheless be detected. One of the drawbacks of this kind of operation arises from the difficulty of choosing operating conditions and of predicting chromatograms from data of the analytical separation. The extrapolation is not straightforward, and parameters such as column length, concentration of the feed and sample size may be of crucial importance. Therefore, the overloaded separation presented in this work is probably not the best possible, but we lack theoretical analysis to adjust the parameters. The solution to these problems may be found in the numerical modelling of the chromatographic process under these conditions of high concentrations, *i.e.* non-linear chromatography modelling, owing to the non-linear character of the set of partial differential equations. From the point of view of numerical resolution, this modelling is not easy and constitutes nowadays a challenge for mathematicians. A recent review on this topic may be found in ref. 5. These numerical models have to be fed with the appropriate description of the basic non-linear interaction between solutes and stationary phase whose knowledge, in the case of proteins and ion-exchange resins, is still under progress in the recent literature [6,7]. The stoichiometric displacement model (SDM), established by Velayudhan and Horváth [8], is probably the most appropriate. When the coupling of these two models, *i.e.* the chromatographic process and the SDM model, has been achieved, this kind of overloaded separation may be easily optimized and should lead to the development of ion-exchange liquid chromatography as an efficient technique for the preparative purification of proteins.

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