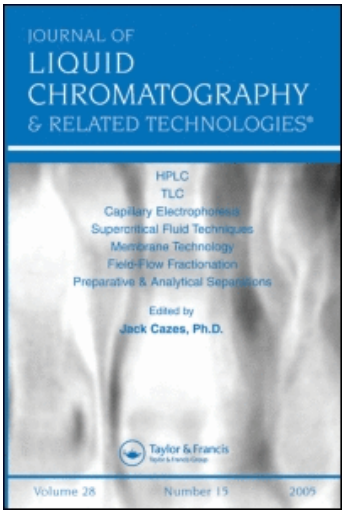

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PREPARATIVE HPLC OF SOYBEAN TRYPSIN INHIBITOR USING LARGE PARTICLE DIAMETER SUPPORTS

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ABSTRACT

Two systems were developed to purify Soybean Trypsin Inhibitor (STI) using anion exchange chromatography. Both systems demonstrated that large diameter particles (30 and 55 μm) could be used effectively for protein purification. Preparative samples of 455 mg and 7.2 g were processed to yield highly purified products. Preparative purifications were achieved either on an analytical instrument with an analytical (0.46 x 30 cm) column or on a large scale system with a preparative (4.8 x 50 cm) column. Sample introduction by frontal loading appeared to lead to some protein-protein displacement, thus allowing for some pre-gradient fractionation and enrichment of the more strongly retained STI on the column. This is referred to as an "overfed" system. Throughput is discussed and used to evaluate the different systems. An apparent lack of resolution in preparative scale chromatograms did not necessarily indicate an absence of fractionation. It was possible to isolate protein products of high purity with high throughput values using larger diameter supports.

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INTRODUCTION

In recent years, high performance ion exchange chromatography (IEC) has been successfully employed in the separation of ionic solutes ranging from inorganic and small organic ions to biological macromolecules such as polynucleotides and proteins. High performance IEC systems are substantially faster than the conventional gel-type columns and have much higher resolving power. For example, 10 to 100 fold purification of proteins and polynucleotides in 30 minutes in a single pass through a high performance column is common.

This success in rapid, small scale analytical separations has led to increasing interest in high performance ion exchange chromatography as a preparative technique; the logic being that by increasing the size of high performance analytical columns, it will be possible to scale-up separations. This is advantageous because it would be possible to work out separation conditions on small analytical columns and then apply them to preparative columns that have identical properties. The goal in preparative chromatography, however, is different than in analytical separations. Whereas it is often important in analytical separations to resolve the components of a mixture as completely as possible, the goal in preparative separations is generally the purification of a single component of a mixture. If the objective is to achieve the separation with minimum system requirements, it is easy to see that a column which only has to resolve one of the components of a mixture might be different than one that has to resolve all the components of a mixture. The same

logic would apply to column operation. Conditions and protocol required for the separation of a single component in a mixture could be very different than those required for the separation of a large number of components. These examples illustrate that analytical and preparative separations may be quite different.

The objective of most preparative separations is to purify the largest amount of material in the shortest amount of time at a minimum cost, i.e. to maximize throughput while holding cost at a minimum (1). The cost of solvents, instrumentation and columns all influence separation economics. Numerous techniques have been examined which allow minimization of separation costs. For example, sorbent particle size is an important variable in separation economics. It is apparent that the use of microparticulate analytical media will make a large contribution to separation costs and is to be avoided for economic reasons. Variables that influence throughput and their impact on system economics have also been examined (1). Throughput can be increased by decreasing either the cycle time or the purity of the product. As the purity is allowed to decrease, the throughput will increase. Although this may seem counterproductive, throughput may be more important than higher purity in the early stages of a multi-step purification. Varying gradient slope or flow rate are also acceptable ways to increase throughput by decreasing the cycle time.

Still another technique for increasing throughput is to overload the column. If the mass loaded onto the column is increased and the cycle time and purity are maintained, the throughput will increase.

The mass of sample needed to overload a column is a function of the mixture being separated and does not have a direct relationship to the amount of protein needed to saturate the column. In cases of extreme overloading, the entire length of the column may be saturated with protein. Complete saturation of a column will be referred to here as "overfeeding". Although overloading or overfeeding a column is accompanied by a loss of resolution, the question in a given separation is whether the increase in throughput outweighs the loss in resolution. This paper will focus on overfeed loading and how it effects the throughput and resolution on large diameter sorbents.

Several recent publications have examined the issue of optimal particle size sorbents for preparative HPLC (2-5). Although small particle sorbents are widely used in analytical separations, they have some negative features in preparative chromatography. Since cost is inversely related to particle size, it is more expensive to use smaller particle size sorbents. Small particle sorbents also require higher operating pressures which is undesirable in a large system. On the positive side, smaller particles give less band spreading and have inherently greater resolving power. The central issue is the cost-benefit ratio. The particle size selected must ultimately be based on a balance of sorbent cost against resolving power. Experience has indicated that particles of approximately 20-30 μm size generally have the best compromise between cost and performance. In some cases, particles of greater than 30 μm sizes have shown good resolution in preparative HPLC (6,7). A secondary objective of this study was to further examine the utility of 30 and 55 μm particle diameter sorbents.

MATERIALS AND METHODS

Proteins

Two forms of Soybean Trypsin Inhibitor (STI) were used for this study (Sigma Chemical Co., St. Louis, MO, USA). Purifications were performed on STI Type II-S (crude soluble powder, T9128). STI Type I-S (chromatographically prepared, T9003) was used for comparison.

Electrophoresis standards were obtained from Pharmacia Fine Biochemicals (Piscataway, NJ, USA). SDS-Polyacrylamide gel electrophoresis was performed on the crude sample and a selected fraction according to the method of Laemmli (8).

Buffers

Buffers were prepared from ultrapure Tris (hydroxymethyl)-aminomethane (Boehringer-Mannheim Biochemicals, Indianapolis, IN, USA) and AR grade NaCl (Mallinckrodt, Inc. Paris, KY, USA).

Column and Supports

The overfed system used a 0.46 x 30 cm analytical column packed with Synchrom AX-200, 55 μm anion exchange material (Synchrom, Inc. Lafayette, IN, USA). The column was dry packed using the method of Snyder and Kirkland (9).

A 4.8 x 50 cm Magnum 40 column, dry packed (9) with approximately 350 g of 30 μm anion exchange support, was used as the preparative column (a gift from Whatman, Inc. Clifton, NJ, USA).

Synthesis of the 30 μm Anion Exchange Sorbent

A 750 g quantity of the 30 μm Serva Si-200 (Serva Fine Biochemicals, Inc. Garden City Park, NY, USA) support material was coated with a 5% methanolic solution of polyethyleneimine-6 (PEI, Polyscience, Inc. Warrington, PA, USA) and crosslinked with a 10% methanolic solution of 1,4-butanediol diglycidyl ether (Aldrich Chemical Co., Milwaukee, WI, USA) according to Kopaciewicz et al. (10). All steps and reagents were directly scaled up from the referenced procedures.

Instrumentation

The overfed system used a Pharmacia FPLC® (Pharmacia Fine Biochemicals, Piscataway, NJ, USA) to generate the gradient. Samples were introduced via an Eldex Model B-100-S single piston pump (donated by Eldex Laboratories, Inc. San Carlos, CA, USA). Absorbance at 254 nm was monitored with an Altex model 153 detector equipped with a preparative flow cell (Anspec, Inc. Ann Arbor, MI, USA).

Preparative chromatography using the 4.8 x 50 cm column was performed with a Milton Roy Milroyal D pump (W. J. Wadsworth and Associates, Inc. Arlington Heights, IL, USA). A low pressure gradient mixer (LP-50) and a Chrom-a-trol gradient programmer were gifts from Eldex Laboratories, Inc. Absorbance was monitored at 254 nm by splitting 1% of the column effluent to an Altex model 153 UV detector equipped with a preparative flow cell. Conductivity was monitored via an AN400 Ion Chromatograph (Anspec).

Analytical chromatography was performed using an LDC Constametric I and IIG system with a Gradient Master (Laboratory Data Control, Riviera Beach, FL, USA). Proteins were detected at 254 nm with the Altex model 153 UV detector.

RESULTS

Analysis of Crude STI Sample

Electrophoresis (SDS-PAGE) and high performance liquid chromatography on a strong anion exchange (SAX) column were the primary analytical techniques used in the determination of protein purity during these studies. Both analytical techniques indicated that this sample contained approximately 29% STI. Recoveries and relative purification values reported throughout this paper are based on this value. An analytical chromatogram of the crude STI sample on the SAX column is shown in Figure 1.

Overfeed Loading

It is common practice in the determination of adsorption isotherms to pump solute into a column until the entire length of the column is saturated. This frontal loading technique (11) was used to load the 0.46 x 30 cm column packed with 55 μm particle size AX-200 weak anion exchange sorbent. The sample feed stream was directed onto the head of the column through the use of an auxillary pump and the Pharmacia V-7 valve located between the mobile phase mixer and the head of the column. Sample feed was continued until the column

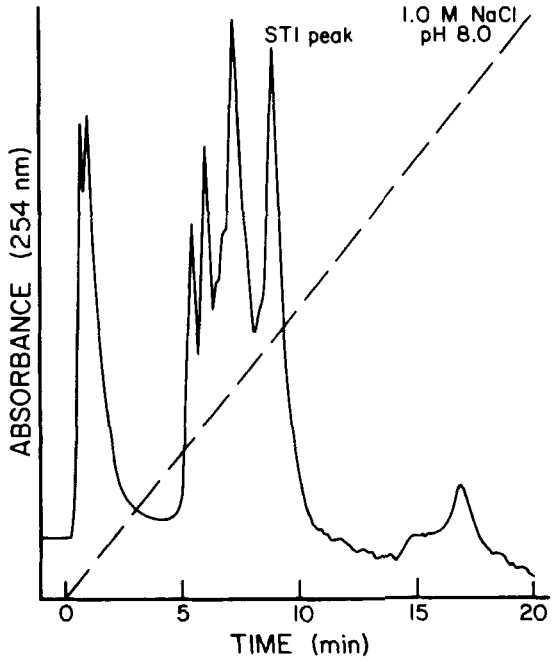

Figure 1. Analytical chromatogram of crude STI sample. One hundred µg of crude STI (T9128) were applied onto a 0.41 x 5 cm, 10 µm, weak anion exchange (WAX) column for analysis. A 20 min gradient from 0 to 1.0 M NaCl in 0.02 M Tris (pH 8.0) was used. The flow rate was 0.5 ml/min and detection was at 254 nm. The peak for STI is indicated.

was saturated and sample components that would normally adsorb began to break through. This sample loading technique will be referred to henceforth as "overfeeding".

In the first preparative run, 455 mg of crude STI at a concentration of 10 mg/ml were pumped into the column in 36 minutes at 1.25 ml/min. Saturation was indicated by a step in the baseline due to protein breakthrough (11) during the loading process (Fig. 2). The size of this step is dependent on the protein concentration in

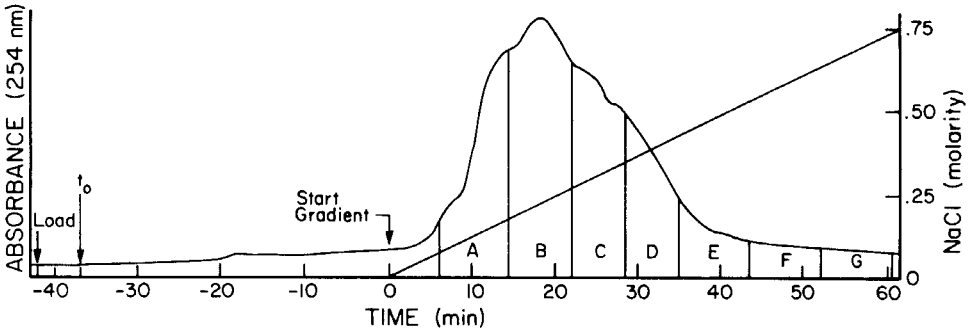

Figure 2. Preparative chromatogram of crude STI sample using the overfed system. Four hundred and fifty five mg of crude STI (T9128) were loaded directly onto the 55 µm AX-200, 0.46 x 30 cm column. The starting points of protein loading and the gradient are indicated as is the void time for the column (t_o). The flow rate for loading was 1.25 ml/min and 0.5 ml/min for the gradient. The gradient ran for 80 min from 0 to 1.0 M NaCl in 0.02 M Tris (pH 8.0). Fractions A through G were collected in approximately equal volumes.

the sample feed stream. Following loading, the column was eluted at 0.5 ml/min with a gradient ranging from 0 to 1 M NaCl in 80 minutes. The total cycle time, including 4 minutes for recycling the column, was 120 minutes. The static loading capacity of this column for crude STI was 320 mg.[1] Therefore, the 455 mg of protein used in the preparative run were sufficient to saturate the surface. In overfeed loading, a pre-gradient separation results from the elution of weakly retained material from the column. Thus, STI composed a higher percentage of the material on the column after loading than it did in the crude sample feed (13).

It appeared in the elution profile (Fig. 2) that little resolution of the sample components was achieved when the overfed

[1]Static loading capacity of a column is a computed value based on the maximum amount of crude STI that a unit mass of sorbent will adsorb in a batch assay (12).

column was eluted. However, HPLC analysis of the collected fractions (A-G) indicated significant purification of STI (Fig. 3). The fact that no STI was found in fraction G may have been due to displacement of STI by later eluting peaks which reduced the tailing of the STI peak. Fraction D contained the highest percentage of STI and was dialyzed and lyophilized for further use as a chromatographic standard. Since Fraction C had a higher percentage of STI than the crude sample, it would be economically beneficial to recycle this fraction.

A comparison of Fraction D with the type I-S STI from Sigma (Fig. 4) indicated that the isolated STI was at least as pure as the high grade commercial STI. Further analysis of Fraction D with SDS-PAGE (Fig. 5) using a 15% acrylamide gel and three different protein concentrations showed a substantial reduction in lower molecular weight species relative to the crude sample. When the SDS gel was scanned using a densitometer, the isolated STI appeared to be at least 95% pure. Table 1 summarizes the results of the overfed column separation in terms of recoveries and yields. Since the highest purity material was needed and crude STI is inexpensive, only fraction D was used in calculating throughput and fractions A, B, C, E and F were discarded. Recycling fractions A, B, C, E and F would have increased the yield and throughput.

Non-overloaded System

A second mode of preparative chromatography examined involved the use of a large column under non-overloaded conditions. In this

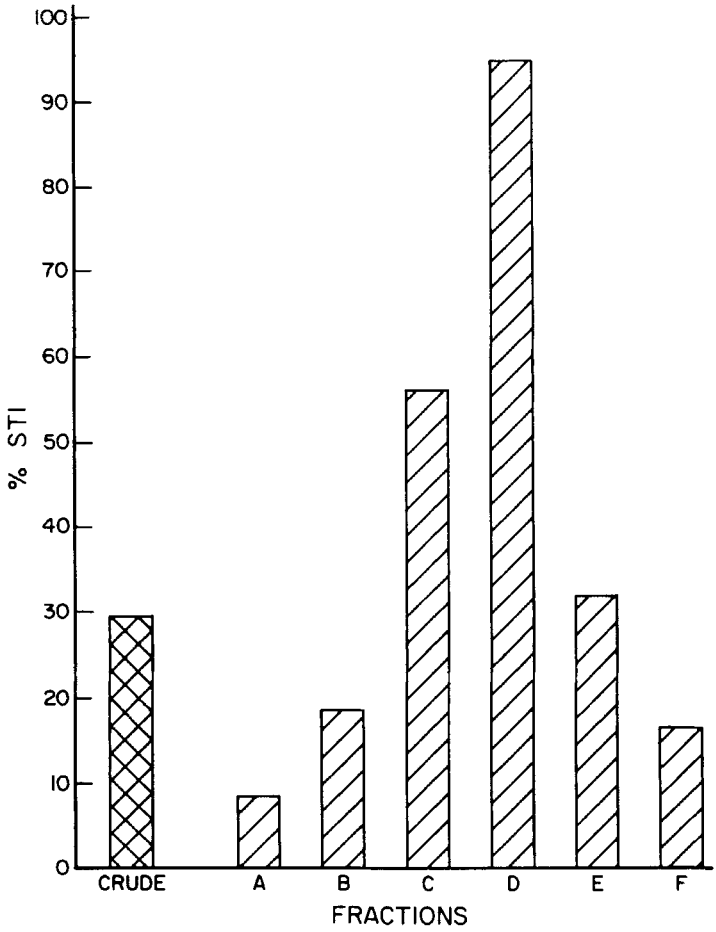

Figure 3. Percentage of STI in each fraction. The percent of STI in the crude sample and in each fraction was plotted as a bar graph. Fraction G contained no measurable amount of STI and was not plotted. The percentages were determined by the ratio of the mass of the STI peak compared to the mass of the entire fraction.

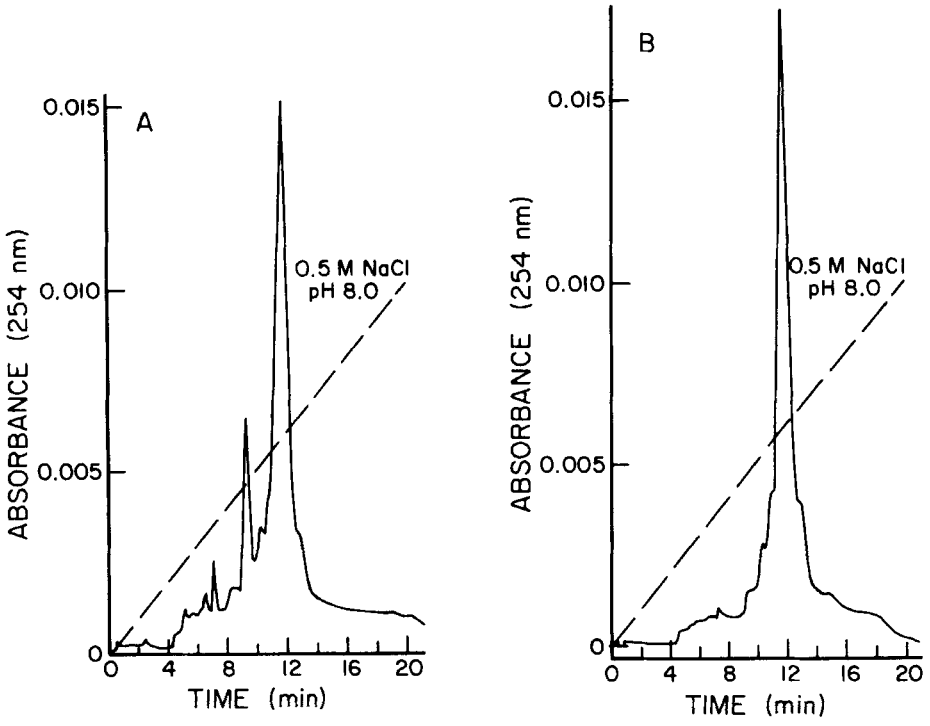

Figure 4. Comparison of purified STI from Fraction D to the high grade STI. Type I-S (T9003) STI from Sigma was chromatographed under identical conditions as Fraction D to compare the purity. (A) 240 µg of Type I-S STI were injected onto a 0.41 x 5 cm WAX column. The gradient ran from 0 to 0.5 M NaCl in 0.02 M Tris (pH 8.0) in 20 minutes. The flow rate was 1 ml/min. (B) 240 µg of purified Fraction D STI were injected onto the WAX column under the same conditions as in (A). This weak anion exchange column was slightly different from that used in Figure 1, hence, the different gradient.

case, the elution profile more closely parallels the analytical separation. Therefore, it becomes easier to extrapolate to preparative separation conditions from the analytical values. A 4.8 x 50 cm column was packed with PEI-coated 30 µm Serva Si-200 Silica (250 Å pore diameter). The total loading capacity of this column was 59.6 g of crude STI as determined by static assay (12).

TABLE 1

Purification Characteristics of the "Overfed" System[1]

Sample Load	STI in Sample Load	STI in Fractions A-F	STI in Fraction D	Mass of Fraction D	Purity[2] of Fraction D
455mg	134mg	110mg	43mg	45mg	95%

Recovery[3] of STI	Yield[4] of STI	Throughput[5] mg D/time
82%	39%	0.38mg/min

[1]The column used in this study was 0.46 x 30 cm packed with 55 μm, AX-200.
[2]Purity = Mass of STI in Fraction D/Mass of Fraction D.
[3]Recovery = STI in Fractions A-F/STI in load.
[4]Yield = STI in Fraction D/STI Recovered.
[5]Throughput = Mass of STI in Fraction D/Cycle Time.

Therefore, the 7.2 g sample load used in Figure 6A was only 12% of this saturation value. Detection was performed by splitting the stream just beyond the column and passing 1% of the effluent through the detector flow cell. Figures 6B and 6C show the analytical SAX chromatograms of the crude and purified STI obtained from this 4.8 x 50 cm column. The collected STI fraction is seen to still contain some impurities (Fig. 6C). After lyophilization of this fraction, 2.6 g of material were recovered. The total time for sample loading and gradient elution was 55 minutes. Although product purity was lower in this case, the throughput was 47 mg/min. At a flow rate of 100 ml/min in this system versus 0.5 ml/min for the overfed system, the amount of mobile phase consumed was almost 60

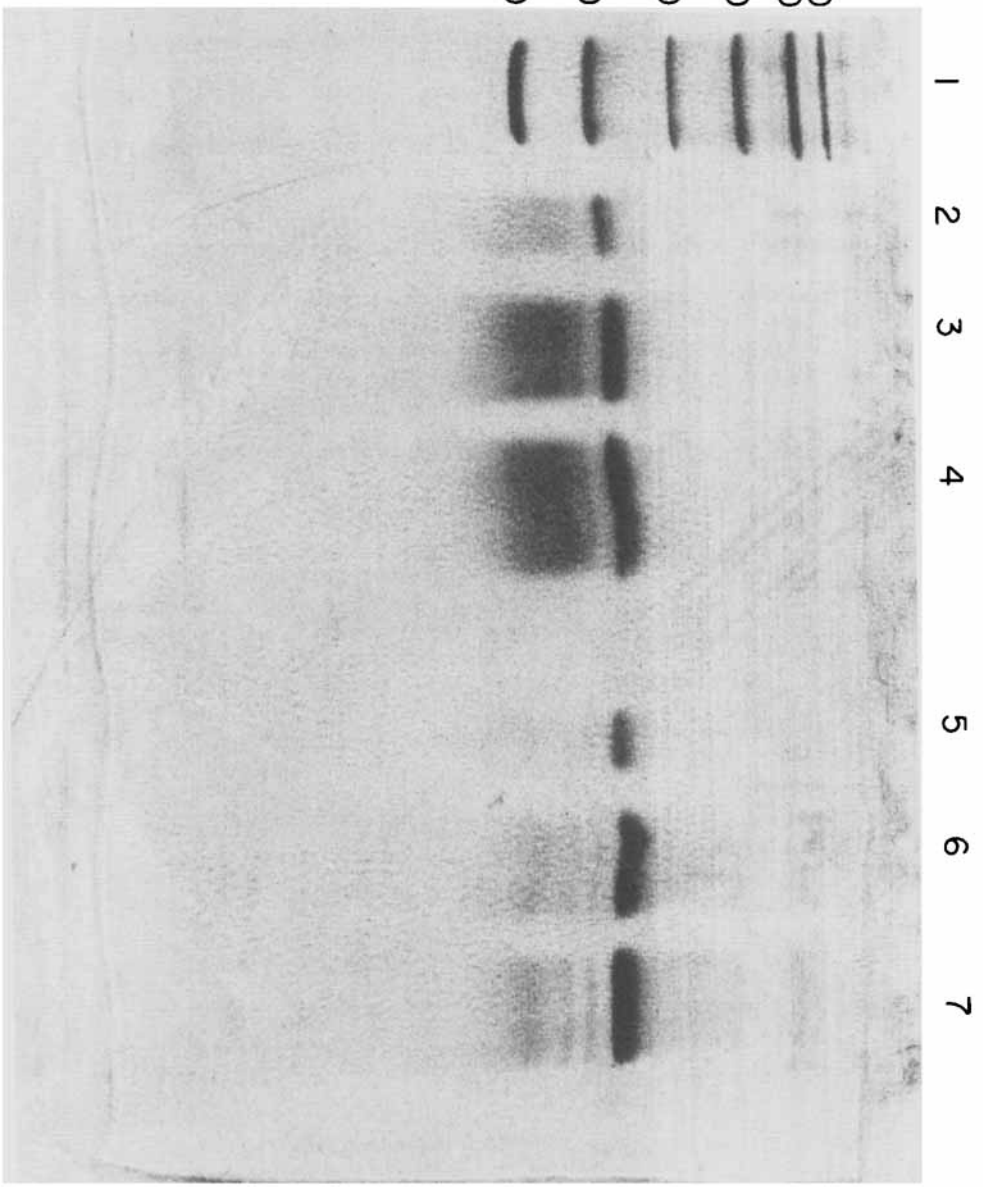

Figure 5. SDS-PAGE analysis of the initial sample and Fraction D. Electrophoresis was performed in a 15% acrylamide gel under denaturing conditions with sodium dodecyl sulfate and β-mercapto-ethanol. Lane 1 contained the marker proteins. The bands were from highest to lowest molecular weight: Phosphorylase b, Bovine Serum Albumin, Ovalbumin, Carbonic Anhydrase, STI and α-Lactalbumin. Lanes 2-4 were the crude STI at amounts of 1, 5 and 7.5 μg, respectively. Lanes 5-7 showed Fraction D also at amounts of 1, 5 and 7.5 μg.

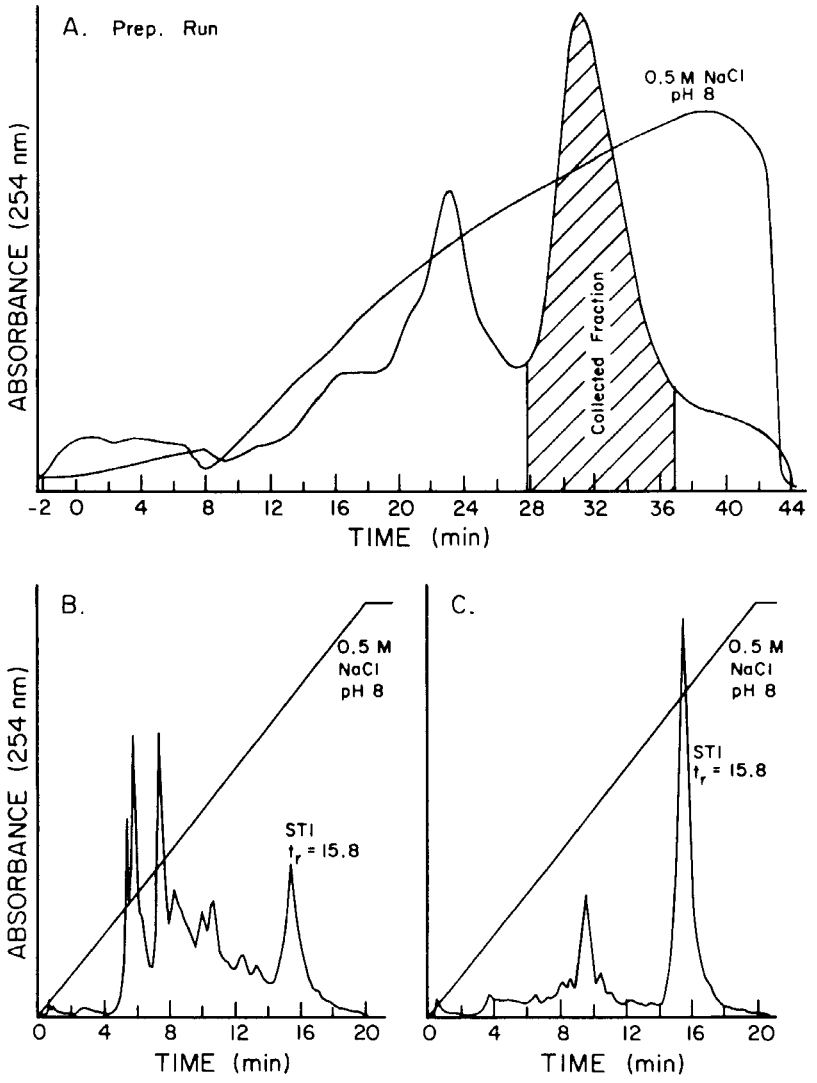

Figure 6. The preparative isolation of soybean trypsin inhibitor. A multigram sample of crude STI (Sigma) was fractionated on the preparative system (A). Seven hundred and twenty ml of 1% (w/v) protein solution were loaded onto the column. Absorbed protein was eluted with a 30 min linear gradient from 0.01 M Tris (pH 8.0) to 0.5 M NaCl in 0.01 M Tris (pH 8.0) at a flow rate of 100 ml/min. The fraction collected is indicated by the hatched region. Chromatograms "B" and "C" are analytical separations (0.41 x 5 cm column packed with quaternized, PEI-coated silica) before and after the preparative fractionation. Elution conditions were the same as in Figure 4.

times greater for the preparative column. At a larger sample load, throughput for the preparative system would still increase without a substantial increase in solvent consumption.

Throughput Evaluation

Throughput is dependent on several parameters (1). The one evaluated in this study was the mass of sample loaded relative to the total volume of the column. This was achieved using non-overloaded and overfed columns and by specifying throughput in terms of the mass of sample purified/unit time in a ml of column volume, i.e., mg/min/ml.

It has been noted that a 10 mg protein load on a 0.41 x 25 cm column packed with 10 μm ion exchange sorbent will cause a 10% decrease in resolution (11).[2] If a 10 mg sample of crude STI were loaded onto this column, 29% or 2.9 mg of the sample would be pure STI. Assuming a cycle time of 30 min, 100% recovery of STI in a single fraction and greater than 95% purity of the collected STI, this 0.41 x 25 cm column would have a throughput of 0.029 mg/min/ml. Even this optimistic value is well below the throughput of 0.075 mg/min/ml obtained with the overfed column (Table 2). In contrast, the large preparative column (4.8 x 50 cm) had a throughput of 0.052 mg/min/ml with much less purity.

Specific Solvent Consumption

Solvent consumption was evaluated in terms of the specific volume of solvent needed to purify a unit mass of solute (ml

TABLE 2

Comparison of Throughput Values for Three Different Systems

System	Mass mg	Time min.	Throughput mg/min	ml/Col ml	Throughput/ml column mg/min/ml
Analytical 0.41 x 25 cm	3	30	0.10	3.3	0.029
Overfed 0.46 x 30 cm	45	120	0.38	5.0	0.075
Preparative 4.8 x 50 cm	2600	55	47	905	0.052

[2]A 10% decrease in resolution caused by sample loading is usually considered to be the onset of overloading.

solvent/mg protein). In the hypothetical analytical system described above (0.41 x 25 cm), 30 ml of solvent would be consumed in a 30 min cycle with 2.9 mg of protein recovered. This would result in a specific solvent consumption of 10.3 ml/mg of protein. The overfed system required 95 ml for a full cycle and produced 45 mg of protein giving a specific solvent consumption of 2.1 ml/mg. Finally the preparative system used 5500 ml per cycle and yielded 2600 mg of protein for a specific solvent consumption value of 2.1 ml/mg. As previously mentioned the material purified with the preparative system was not as pure as the overfed system. If the purity was equal, the mass of protein (mg) recovered from the preparative system would be less and the specific solvent consumption would increase. Thus, it is seen that specific solvent consumption in the overfed system would be approximately five times less than in the analytical system and slightly less than the preparative system.

DISCUSSION

In this study, two systems were described to purify STI from a crude soluble extract. The preparative system consisted of a 4.8 x 50 cm column packed with 30 μm weak anion exchange sorbent. The high flow rate of the system made the loading step short relative to the entire cycle time. Hence, in the preparative system this portion of the cycle time could not be significantly reduced in an effort to increase throughput. Since the column was loaded to 12% of its full capacity, throughput in mg/min/ml was higher than the hypothetical analytical system, but lower than that of the overfed system. The disadvantage of this preparative system was that the protein product was of lower purity than the STI from the overfed system.

In the overfed system, the amount of sample processed was greater than the capacity of the column. This is possible because some sample components do not adsorb to the column. Relative to the preparative system, the loading time for the overfed system occupied a large fraction of the cycle time (30%). It seems likely that this portion of the cycle time could be reduced without effecting the separation, thus increasing throughput.

Both systems used large particle diameter supports (30 and 55 μm) for the separation of STI. Large diameter supports provided less resolution than the 6.5 μm analytical supports but had the advantage of very little back pressure and therefore, the option of a wider range of flow rates. This would allow for flow programming during elution and faster flow rates during the loading and recycle

steps. Fast recycle and loading steps are desirable for higher throughput. Large particle diameter supports are also considerably less expensive. The 30 and 55 μm particles are eight to sixteen times less expensive than 6.5 μm particles with the same support chemistry.

The overfed system had the highest throughput of the systems examined with a product throughput 2.5 times greater than the analytical system. In a single two hour run, 15 times more protein was purified with the overfed system than could have been obtained from the analytical system (Table 2). In order to produce an equivalent amount of protein, the analytical system would have required 15 cycles and five hours. Reducing the cycle time of the overfed system would have made the difference in throughput even greater.

The results from overfeed loading showed that the entire length of a column could be loaded with sample and still have separation of the components. As a result of heart-cut collection, high purity material was obtained even in this overfed system (Fig. 3). Recycling enriched fractions from the leading and tailing edges of the STI peak would have increased the percentage of STI in the feed stream and therefore increased throughput and recovery. If a larger (1.5 x 30 cm) column was used, 4.55 g of crude sample could be processed and 430 mg of STI could be collected in pure form per 90 min cycle on an analytical instrument. In only 10 runs, an analytical instrument with a pumping capacity of 10 ml/min could process 45 g of sample per day.

In addition to the throughput term, specific solvent consumption (ml/mg) should also be considered when evaluating performance in preparative systems. Both terms will influence the cost of products and also the procedures used for production. In many instances, specific solvent consumption will be inversely related to throughput. For example, when the throughput is increased by shortening the cycle time with the same flow rate, specific solvent consumption will decrease. Also, as the purity requirements increase throughput decreases and specific solvent consumption increases.

It is important to note for the optimization of solvent consumption that specific solvent consumption is in units of volume/mass. Although flow rate and cycle times may be altered to increase throughput, specific solvent consumption will only change when one of the variables altered changes the total cycle volume. As the flow rate is increased for a faster recycle step and therefore higher throughput, specific solvent consumption will not change if solvent volume remains the same for recycling. In contrast to recycling, flow rate reduction during the gradient decreases specific solvent consumption. Since it is reasonable to assume that the volume of solvent required to recycle a column is a constant and that it is possible in some cases to reduce the flow rate during gradient elution, it appears that the gradient is the most likely variable with which specific solvent consumption can be manipulated. However, reduction of solvent consumption has limits. For overfed systems, protein solubility may become a limiting factor when trying to reduce the flow rate and optimize specific solvent consumption. In general,

throughput and specific solvent consumption will be optimized by using shorter gradients at reduced flow rates and rapid recycling.

This study has shown that non-overloaded systems may have as much as five times greater specific solvent consumption than overfed systems. By using an overfed system and by progressively reducing column size throughout a purification scheme, specific solvent consumption and throughput can be optimized. As was the case with the small analytical column, preparative systems will have a higher throughput and a lower specific solvent consumption when operated in an overloaded or overfed mode.

CONCLUSION

It may be concluded that substantial resolution of components in a protein mixture can be achieved in columns where the entire length of the column is saturated with sample. The advantage of overfeeding columns during the loading process is that greater throughput may be obtained. When data for throughput and specific solvent consumption are taken together it is seen that an overfed column is capable of purifying 2.5 times as much protein with 1/5 the specific solvent consumption as a column of comparable size that is not overloaded. Assuming solvent costs to be directly proportional to solvent volume, overfeeding columns is of clear economic superiority in the case examined here.

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