0273-2289/98/73–2/3—0016$12.00

Continuous Purification of Porcine Lipase by Rotating Annular Size-Exclusion Chromatography

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Received March 31, 1997; Accepted March 24, 1998

ABSTRACT

Crude porcine lipase (triacylglycerol lipase, EC 3.1.1.3) was purified in a single-stage chromatographic process. The purification was accomplished in a batch, as well as in a continuous system. Two types of size-exclusion packing materials (Sephadex and Sephacryl) were used. The average x-fold increase in purity, and the average recovered activity in the batch Sephadex and Sephacryl experiments were 13.6 and 89.7%, and 34.2 and 98.8%, respectively. The average x-fold increase in purity and the average activity recovered in the continuous Sephadex and Sephacryl experiments were 27.1 and 82.5% and 16.2 and 89%, respectively. Flow visualization experiments were carried out by tagging the protein to be separated with a fluorescent dye. The results from these experiments are also reported in this article.

Index Entries: Porcine lipase; continuous chromatography; size-exclusion chromatography; lipase purification.

NOMENCLATURE

F, flow rate, mL/min; H, plate height, cm; h_i, absorbance, dimensionless; L, packing height, cm; N, number of plates, dimensionless; t_R, peak retention time, min; R_S, resolution, dimensionless; V_i, elution volume, mL; V_R, peak retention volume, mL; σ, standard deviation, mL.

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INTRODUCTION

Lipases are produced by plants, animals, and microorganisms *(1)*, and as such are widely distributed in nature. As enzymes, lipases have the ability to catalyze a variety of chemical reactions. Their availability and diversity as a biocatalyst make them attractive to a number of industries. Crude lipases, which often contain less than 1% lipase, are commercially available at low prices; however, pure enzymes are preferred for industrial applications *(2)*. The problem is that considerable effort must be expended to produce pure lipase from crude lipases obtained in nature. Most studies of lipase purification in the literature utilize a multiple-stage purification, including precipitation, followed by various types of batch chromatography. Of these studies, about seven have reported both total protein concentration and enzyme activity *(3–9)*. A rotating annular system, first conceived by Martin *(10)*, and recently studied by Bloomingburg et al. *(11)*, Begovich and Sisson *(12)*, and Canon et al. *(13)*, can be used to make a batch process continuous. Experiments were conducted on an ion-exchange chromatograph *(12–14)*, and data were obtained on the separation of nickel and cobalt salts. The Oak Ridge group studied the effect of various parameters on column performance, and were able to operate their unit at high pressures. The authors' objective was to separate lipase by size-exclusion chromatography in a continuous rotating unit. Since one of the aims of the study was to carry out flow-visualization studies, the entire unit was built of plexiglass. The continuous unit was designed and fabricated by this group. The specific objectives of the research were to evaluate the effect of various parameters, such as solvent flow rate and rotation rate of the annulus, on the purification of lipase. Two different packings were studied. The packing was introduced in the annular region, and the effect of packing height on elution profile was also determined. Prior to conducting continuous experiments, studies were carried out in a batch column. The results from the batch studies were used in optimizing the performance of the continuous unit. This article presents results obtained from the batch and continuous purification units, using two different packings. Results from flow-visualization studies, which were helpful in optimizing the performance of the continuous unit, are also presented.

MATERIALS AND METHODS

Materials

The starting material, crude porcine lipase, was acquired from Sigma (St. Louis, MO; Cat. No. L 3126). The enzyme was refrigerated until further

use. Sephadex (Cat. No. G-75-120, with a fractionation range of 3000–80,000), and Sephacryl (Cat. No. S-100-HR, with a fractionation range of 1000–100,000) were purchased from Sigma. The Sephadex was stored at room temperature, and the Sephacryl was refrigerated until further use. For enzyme activity assay, substrate triacetin (Cat. Number T-5376), a color indicator, thymolphthalein; (Cat. No. T-0626), and a buffer, Trizma Base (Cat. No. T-1503) were also obtained from Sigma. The other necessary materials were obtained from different manufacturers: A.C.S. certified concentrated hydrochloric acid from Fisher Scientific (Fair Lawn, NJ); A.S.C. certified sodium hydroxide pellets from VWR, West Chester, PA; and 200-proof ethyl alcohol from Aaper Alcohol and Chemical, Shelbyville, KY. The materials for the Lowry method were purchased from Sigma in the form of a kit (Cat. No. 690-A). The kit included the following: Biuret reagent, Folin and Ciocalteu's phenol reagent, and a protein standard. Sigma 0.85% sodium chloride solution (Cat. No. 430AG-4) was purchased separately. For the flow-visualization experiments, fluorescein isothiocyanate (FITC) (Cat. No. 4022F), was obtained from Research Organics, Cleveland, OH.

Preparation of Lipase Solutions

Lipase solutions were prepared by dissolving the powder in cold distilled water. The cold lipase solution was then centrifuged at 3500 rpm (1380g) for 5 min. The clear lipase solution was decanted and used in the separation experiments.

Preparation of Size-Exclusion Packing Materials

Dry Sephadex beads were placed in distilled water at room temperature for a period of 24 h. The wet swollen beads were then used in the separation experiments. The Sephacryl came pre-swollen in a 20% ethanol–water solution. These beads were rinsed with distilled water, and then used in the separation experiments.

Measurement of Lipase Activity

A titrimetric method obtained from Sigma was used to measure lipase activity. This method utilizes triacetin as a substrate. The enzyme activity is defined in the following manner: one unit will hydrolyze 1.0 microequivalent of acetic acid from triacetin in 1 h at pH 7.4 and at 37°C.

Total Protein Assay

The protein concentration of solutions were determined by the Lowry method.

EXPERIMENTAL SETUP

A rotating annular chromatography unit was designed and fabricated in this laboratory. A transparent material of construction was preferred in order to troubleshoot the unit, and also to carry out flow visualization studies. The unit, made of plexiglass, was 0.3048 m long and 0.1524 m in diameter, with a 6.35×10^{-3} m annular space. At the bottom of the annular space, 72 holes, 5 degrees apart, were drilled on the bottom cover. These holes allowed the separated products to exit the unit. The holes were fitted with 4.76×10^{-3} m copper tubes.

The packing material in the annulus was supported by a thin layer of glass wool placed in the annular space above the bottom cover. The glass wool caused less dispersion than a stainless-steel screen support. Flow-visualization studies were extremely helpful during this process. A dye, potassium permanganate, was used to follow the flow pattern through the unit, from the feed at the top, through the packing-support material, and finally through the copper tubes at the outlet. The degree of dispersion caused by the supporting material could be determined visually.

For the feed, a 6.35×10^{-3} m tube was run up through the middle of the unit, and was connected by a swagelok union to the rotary fitting. This tube performed two functions. It carried a 3.18×10^{-3} m flexible tube, which carried the feed into the unit, and also functioned as the tap for measuring the system pressure. The rotary fitting allowed the annular chromatograph to rotate without twisting up the connected tubing.

For the mobile phase, a mobile-phase feed tube, 6.35×10^{-3} m in diameter, was connected by a swagelok union to the rotary fitting, which was threaded to the top cover. The system was designed to run both gravity and forced mobile-phase flows. Under gravity-flow conditions, the mobile phase was evenly added to the top of the bed via a shower head. The shower head was fitted with twelve 3.18×10^{-3}-m outlet tubes, one every 30 degrees, and distributed the flow evenly to the top of the bed.

The 3.18×10^{-3}-m feed tube continued through the bottom rotary fitting, and was attached to the shower head. The feed that entered the unit through the rotary fitting in the bottom of the inner cylinder was fed to a specific location on the top of the bed. This was accomplished by fitting the flexible one-eighth in. tubing to a 1.59×10^{-3}-m stainless-steel needle epoxied to the shower head, and submerged below the packing surface. This prevented the feed from getting dispersed in the liquid above the packing.

A timing-belt system was used to drive the unit. The timing-belt pulley was mounted to the top of the unit, and the other end of the belt was connected to a drive shaft. The drive shaft was connected to a motor with a variable resistor, so that the unit could be operated at different speeds.

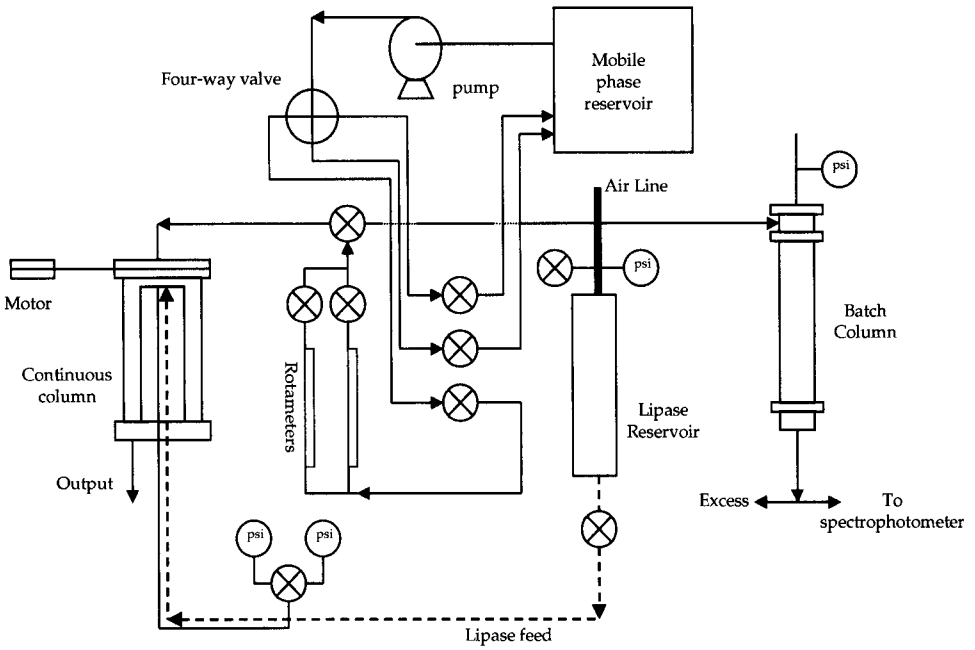

Fig. 1. Experimental Setup.

Figure 1 shows a schematic of the experimental setup. The unit could be operated at pressures up to 239 kPa. Accessory equipment included a 0.02-m^3 mobile-phase reservoir, a one-tenth HP mobile-phase pump, a voltage stabilizer, two Bourdon pressure gauges (0–689 kPa and 0–103 kPa), two Dwyer flow meters (0–50 mL/min and 0–300 mL/min), multiple swagelok valves, and a peristaltic pump to introduce the feed. One additional piece of equipment was needed. When the rotating annular unit was pressurized, the peristaltic pump could not deliver the feed into the unit. This problem was overcome by placing the feed mixture in a 0.025 m (i.d.) acrylic tube. This tube was fitted with two threaded aluminum end caps and a 0–414 kPa pressure gauge. Pressurized air was used to force the mixture through a Gilmont flow meter, and into the pressurized rotating unit.

The batch unit consisted of a 0.0318 m (i.d.) acrylic tube, 0.279 m in length. Both ends of the tube had flanged ends that could be closed off, and the packing material was held in the tube by a 0.076-m circular stainless-steel screen placed between the bottom gasket and the bottom cover. The top cover had inlets for the mobile-phase feed, a 0–689 kPa pressure gauge, and a feed-injection point. The bottom cover had an outlet tube, which was connected to a flowthrough cell of a Spectronic 1001 plus spectrophotometer.

Experimental Procedure

In batch trials, the absorbance of the products from the unit were measured spectrophotometrically at 280 nm. The absorbance values were recorded using Micro-Quant software on a PC. Trials were conducted at different flow rates for the two packings, Sephacryl and Sephadex.

In the continuous runs, the mobile-phase pump was started and set to the desired flow rate. The motor was turned on, and the desired rotational speed was set. Finally, the feed pump was set to the desired flow rate, and turned on. A collection tray, consisting of thirty-six, 5-mL test tubes, was designed in such a way that the diameter of the tray matched that of the annular space. The eluent was collected in the tubes, and analyzed. Two collection trays were made, so that more than one set of samples could be taken for each continuous run. If the corresponding samples in each tray had the same absorbance, the unit was assumed to be operating at steady state.

Once the samples were analyzed, the ones that showed high protein concentrations were tested quantitatively for both protein content and lipase activity. The total protein concentration was determined by the Lowry method. A titration method, using triacetin as a substrate, was utilized to measure lipase activity.

RESULTS AND DISCUSSION

Figure 2 shows a chromatogram of a batch separation using Sephadex size-exclusion packing material. The chromatogram indicates the presence of at least two species within the crude porcine mixture that absorb at 280 nm, and the shoulder present between the two peaks suggests the possibility of a third. Sample fractions were taken at all three peak locations, and tested for lipase activity and protein concentration. Lipase activity was found only in the fractions collected at peak 1. The average x-fold increase in purity, and the average recovered activity for six replicates, as shown in Table 1, were 13.6 and 89.7%, respectively. The average x-fold increase in purity of the lipase is defined as the ratio of the sample specific activity (SA; U/mg of protein) to the SA of the injected mixture. The average recovered activity is defined as the total activity (units) of the sample divided by the total activity of the injected mixture.

Comparison of Figs. 2 and 3 shows the effect of increased packing height. Increasing the packing height from 5.5 in. (0.1397 m) to 10.5 in. (0.2667 m), and keeping everything else constant, increased the retention times of peaks 1 and 2 from 7 and 17 min to 11.5 and 31 min, respectively. In addition to the shift in retention times, the bandwidth of both peaks in Fig. 3 is larger. Band broadening is caused by certain rate processes, such

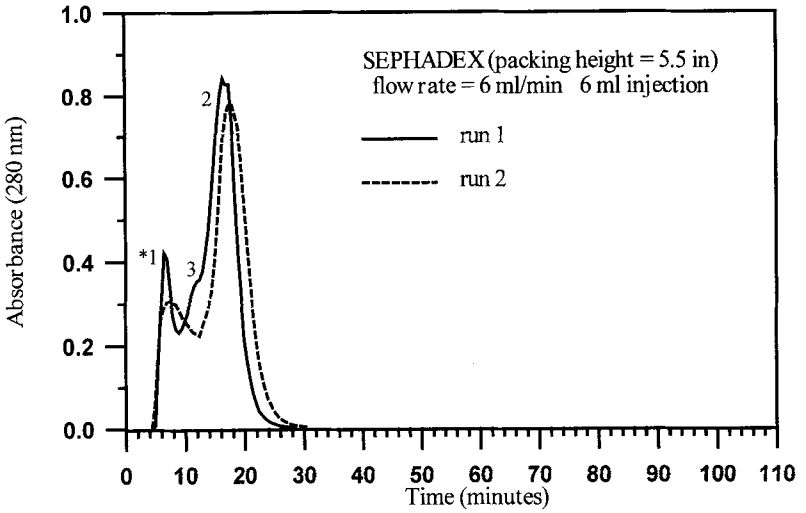

Fig. 2. Chromatogram of batch separation using Sephadex packing material (packing height, 0.14 m; flow rate, 6 mL/min; 6 mL injection).

Table 1
Comparison of Results of Lipase Purification

Run/packing	Total protein mg/mL	Activity U/mL	SA U/mg	x-fold increase	% Recovered activity
Batch/Sephadex	3.96	31.7	8	13.6	89.7
Batch/Sephacryl	2.71	36.25	13.4	34.2	98.8
Cont./Sephadex[a]	1.29	13.75	10.65	27.1	82.5
Cont./Sephacryl[b]	2.15	15	6.98	16.2	89

[a] 1.5 mL/min flow rate, 0.125 rpm.
[b] 2.67 mL/min flow rate, 0.17 rpm.

as eddy diffusion, mobile-phase mass transfer, stagnant mobile-phase mass transfer, and longitudinal diffusion. The influence that some of these processes have on bandwidth is affected by the flow rate and the hydrodynamic conditions of the system.

The effect of mobile-phase flow rate on the elution profile is shown by comparing Figs. 2 and 4. The flow rates in the two cases are 6 and 3 mL/min, respectively. In Fig. 4, peaks 1 and 2 have retention times of 14 and 36 min, compared to 7 and 17 min in Fig. 2. The additional band broadening in Fig. 4 may be attributed to the difference in residence time between the two experiments. However, it is also important to note that a change in flow rate also affects the rate processes.

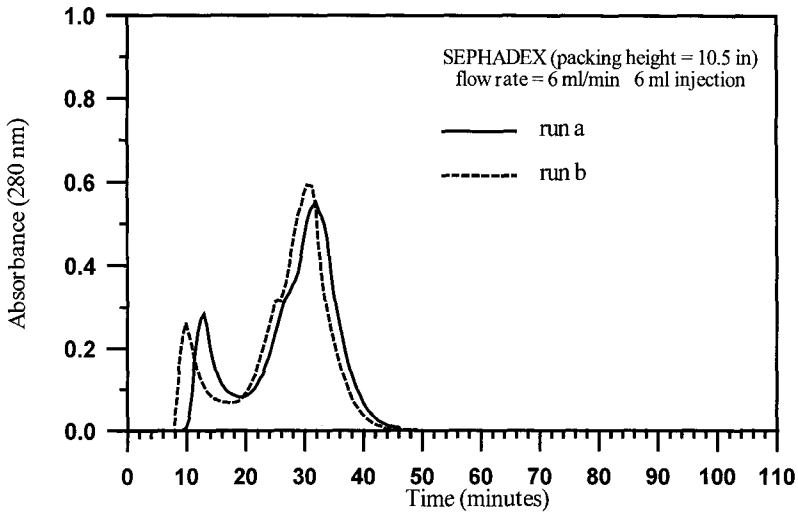

Fig. 3. Chromatogram of batch separation using Sephadex packing material (packing height, 0.27 m; flow rate, 6 mL/min; 6 mL injection).

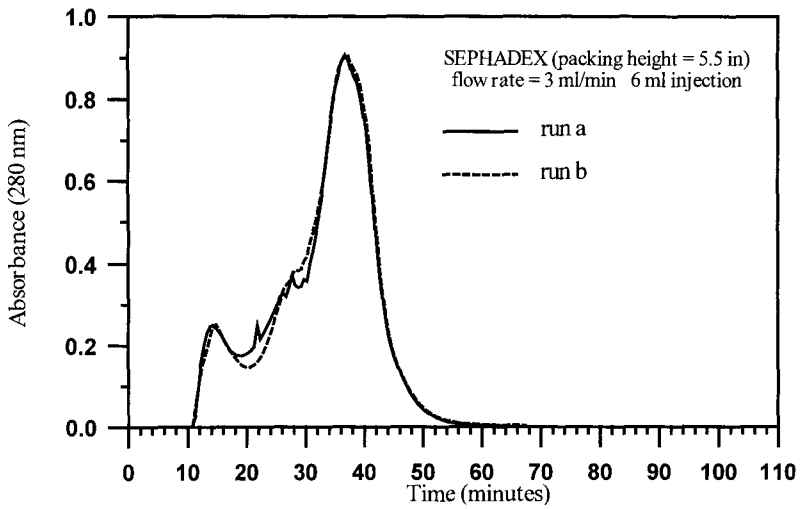

Fig. 4. Chromatogram of batch separation using Sephadex packing material (packing height, 0.14 m; flow rate, 3 mL/min; 6 mL injection).

Figure 5 shows the chromatograms of two batch separations, one using the Sephadex packing material and one using the Sephacryl packing material. This figure shows that, under the same operating conditions, the Sephacryl is able to separate additional components within the mixture. However, the resolution between the lipase peak (peak 1) and peak 2 is not as clear. The appearance of an additional peak (peak 4) in the case of the

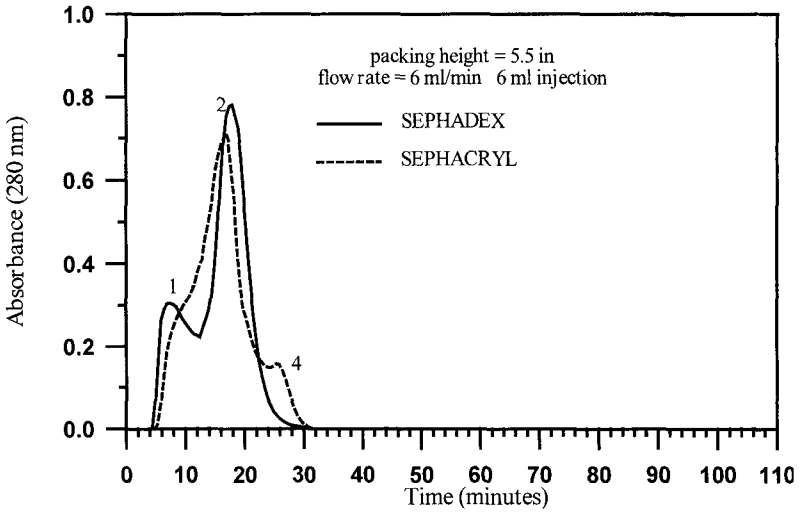

Fig. 5. Comparison of chromatograms using both Sephadex and Sephacryl packing materials (packing height, 0.14 m; flow rate, 6 mL/min; 6 mL injection).

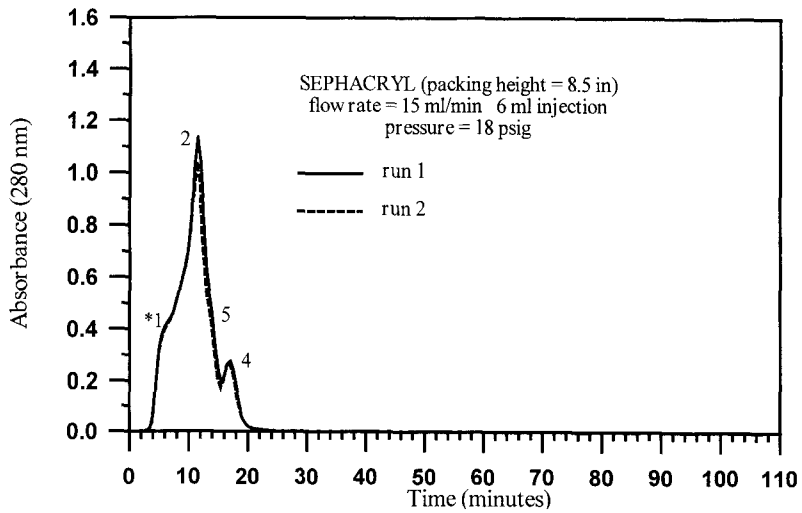

Fig. 6. Chromatogram of batch separation using Sephacryl packing material (packing height, 0.22 m; flow rate, 15 mL/min; 6 mL injection; pressure, 225 kPa).

Sephacryl is caused by its larger fractionation range. Since lipase activity was found only in peak 1, the increased resolution of the second peak, using the Sephacryl, is of little consequence. Figure 6 shows the chromatograms of two replicates, using the Sephacryl packing material under different operating conditions. A slightly better resolution of the peaks is obtained. Sample fractions were collected and tested for lipase activity and

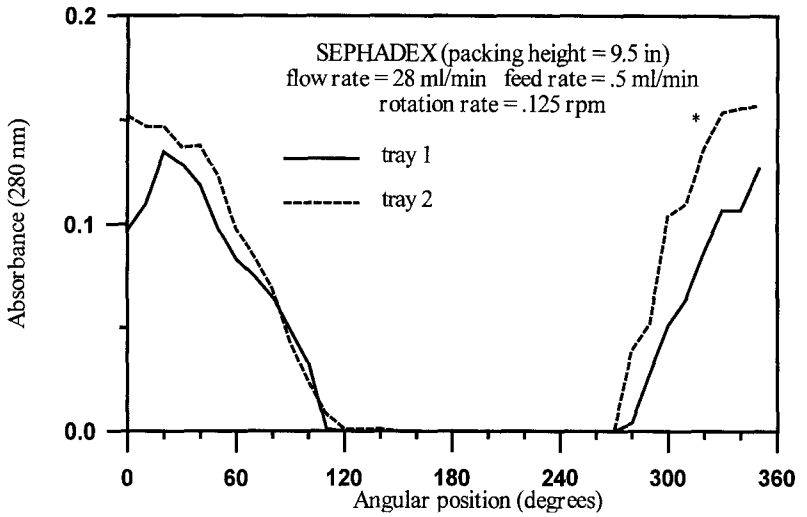

Fig. 7. Chromatogram of continuous separation using Sephadex packing material (packing height, 0.24 m; flow rate, 28 mL/min; feed rate, 0.5 mL/min; rotation rate, 0.125 rpm).

total protein. These tests confirmed that lipase activity was again found under peak 1. As shown in Table 1, the average x-fold increase in purity and the average recovered activity were 34.2 and 98.8%.

Figure 7 shows a chromatogram for a continuous run using Sephadex. The chromatogram shows the concentration of material continuously exiting the system as a function of angular position at the outlet. The reference point is the position directly underneath the feed. Collected material was tested for lipase activity and total protein concentration. Two sets of samples were collected from the unit. The second set of samples was taken 10 min after the first set. These sets of samples are labeled tray 1 and tray 2. Lipase activity was found at approx 310 degrees. As shown in Table 1, the average x-fold increase in lipase purity and the average recovered activity were 27.1 and 82.5%. With Sephacryl packing, lipase activity was found at approx 280 degrees. The average x-fold increase in lipase activity and the average recovered activity in this case were 16.2 and 89.0%, respectively (Table 1).

One of the most important parameters in the continuous separation process is the rotational speed of the unit. Assuming all other parameters are constant, increasing rotational speed beyond a critical point can cause overlapping. Overlapping occurs when two or more components, after separation, exit the column at the same location. Operating a continuous unit under these conditions is undesirable, because the purity of the components is reduced. The critical rotational speed would be the speed at which all of the components exit over 360 degrees.

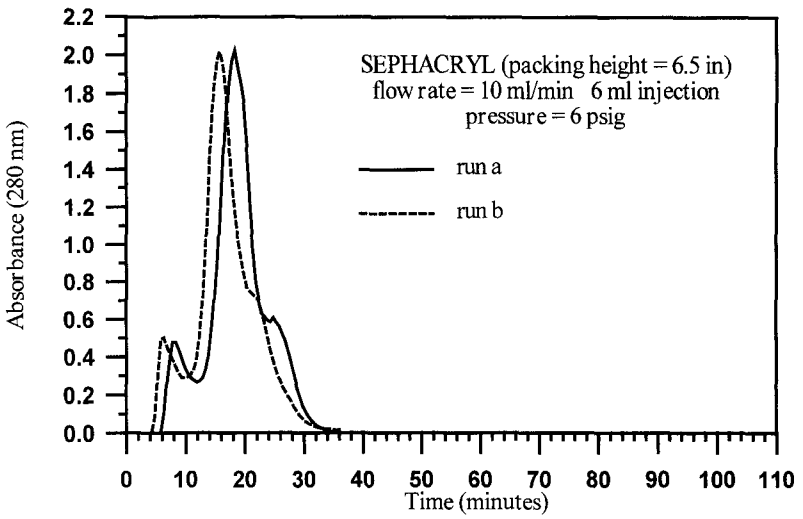

Fig. 8. Chromatogram for separation of dyed protein using Sephacryl (packing height, 0.165 m; flow rate, 10 mL/min; pressure, 143 kPa; absorbance, 280 nm).

A major problem in continuous separation is obtaining feedback when trying to find an acceptable rotational speed, or while waiting till steady state is reached. In a continuous system, samples must be simultaneously taken at every position at the outlet of the unit, and tested for activity. This can be a time-consuming task, and it must be done each time the operating conditions are changed. Batch studies do shed light on some of the operating conditions that should be used in continuous runs, but this is an indirect method that may be time-consuming, as well. An improved method of tuning the continuous process was attempted, and the results are discussed in the following paragraphs.

The mixture to be separated was dyed with fluorescein isothiocyanate (FITC). Initially, experiments were run to ensure that the dye did not affect the size-exclusion separation process. Figure 8 shows the results of a batch separation with the dyed lipase mixture. No change in the separation profile is observed.

The next step was to determine how many of the components in the mixture were marked by the dye. A sample of the dyed lipase mixture was scanned for its maximum absorbance. The solution showed a maximum absorbance at 280 and 490 nm. The absorbance at 280 nm is caused by the protein, and the absorbance at 490 nm (visible region) is caused by the marker that is attached to the protein. Figure 9 shows the results of a batch separation with dyed lipase mixture, under the same operating conditions as in Fig. 8, with the exception that the absorbance is measured at 490 nm. Comparison of these two figures shows that not all of the components

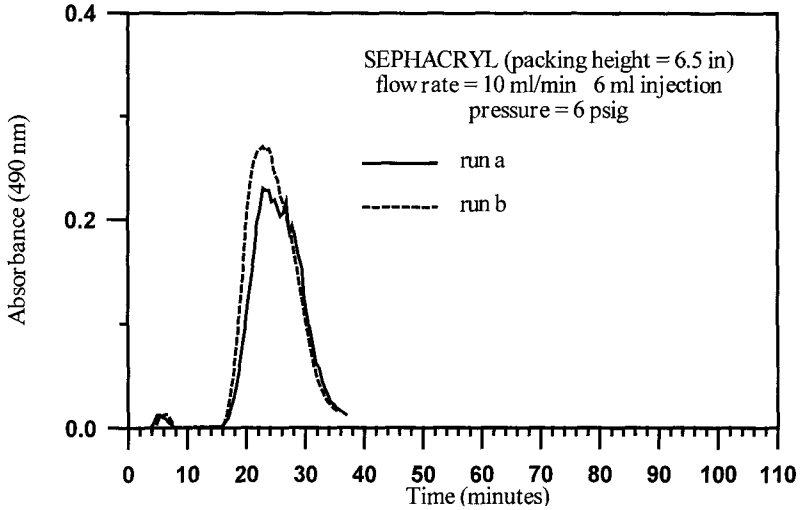

Fig. 9. Chromatogram for separation of dyed protein using Sephacryl (packing height, 0.165 m; flow rate, 10 mL/min; pressure, 143 kPa; absorbance, 490 nm).

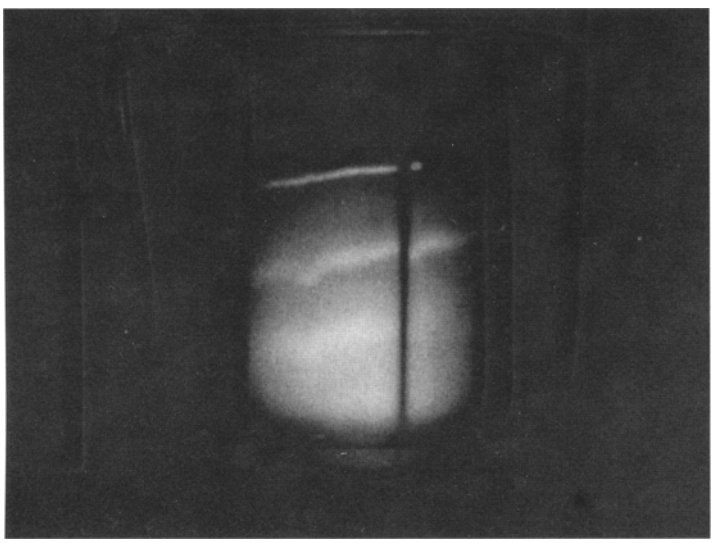

Fig. 10. Photograph of continuous experiment (Sephacryl packing height, 0.19 m; flow rate, 58 mL/min; feed rate, 0.9 mL/min; rotation rate, 0.33 rpm).

within the mixture are marked by the dye. The second component corresponding to the shoulder does not appear at all. Hence, it is still possible to observe the separation process in a continuous unit, even though only the components at larger retention times will be more visible. Figure 10 shows a photograph of a separation using the dyed mixture in a continuous unit with Sephacryl packing. At 0.33 rpm, it is possible to see 3–4

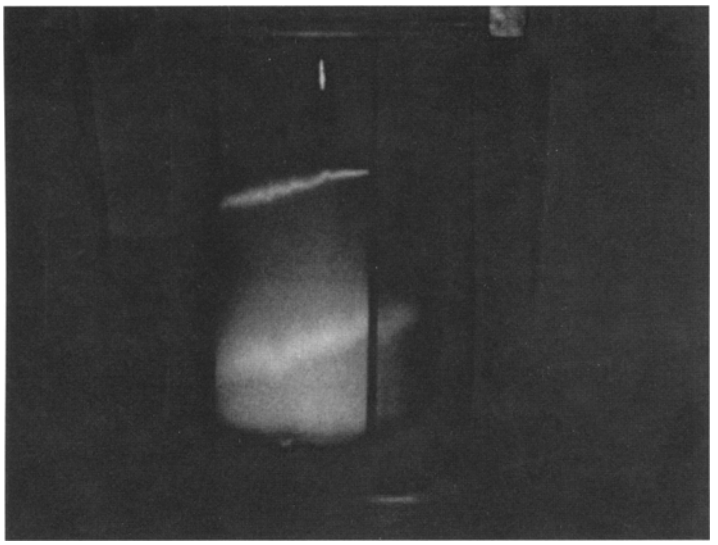

Fig. 11. Photograph of continuous experiment (Sephacryl packing height, 0.19 m; flow rate, 58 mL/min; feed rate, 0.9 mL/min; rotation rate, 0.17 rpm).

wraps, and it is easy to observe that overlapping is a problem. Reduction in the rotation speed by one-half (all other conditions remaining the same) reduced the number of wraps from 4 to 2, as seen in Fig. 11. Thus, it is possible to use this method to experimentally determine the optimum operating conditions for separation. It is also clear from Figs. 10 and 11 that dispersion becomes a problem, especially at higher rotation speeds. Dispersion is also a problem at the exit, because of the presence of the supporting glass wool. One of the problems with running the unit at low rotational speeds is that not all of the packing material may be utilized. If all of the separated components appear within a narrow range, the problem can be solved by installing multiple feed points. However, by a judicious choice of rotation speed and mobile-phase flow rate, it is possible to ensure that the column is well utilized, resulting in a much better resolution. The flow visualization experiments were also valuable in confirming that the retention times in batch experiments roughly translate to retention positions in the continuous unit, under similar conditions of packing height and mobile-phase flow rate.

It is clear from Table 1 that continuous size-exclusion chromatography can be used to purify porcine lipase. With the continuous system, the Sephadex packing seems to result in a higher SA of the protein, compared to Sephacryl packing. This may be attributed to the differences in the operating conditions (Table 1), and the differences explained in the previous paragraph.

Table 2
Comparison of Performance

Run	Packing	Type	Conditions	Peak 1 N	Peak 1 H (cm)	Peak 2 N	Peak 2 H (cm)	Resol^n
Fig. 2	Sephadex	Batch	0.14 m, 6 mL/min, 6 mL injection	28.68	0.487	41.47	0.337	3.822
Fig. 3	Sephadex	Batch	0.27 m, 6 mL/min, 6 mL injection	68.73	0.388	59.91	0.445	3.701
Fig. 7	Sephadex	Continuous	0.24 m, 28 mL/min, 0.5 mL/min, 0.125 rpm	158.44	0.136	99.17	0.218	2.337

$V_R = V_i(t = t_R)$ (t_R defined at peak maximum)
$\sigma^2 = \Sigma[h_i \times (V_1 - V_R)^2]/\Sigma h_i$
$N = V_R^2/\sigma^2$
$H = L/N$
$R_s = [V_{R_1} - V_{R_2}]/[2 \times (\sigma_1 - \sigma_2)]$
Subscripts 1 and 2 denote values related to two different peaks.

Table 2 presents an analysis of the data for both batch and continuous separations. These include the number of theoretical plates (N), height of a plate (H), and resolution. It is clear from Table 2 that the resolution remains more or less constant for the batch separations (irrespective of the column height used). For the continuous process, there is a drop in the resolution, perhaps for the reasons discussed in the previous paragraphs (overlapping and dispersion). Efforts are underway to improve the system, in order to minimize dispersion and overlapping of peaks. However, it is clear from Table 1 that the SA and x-fold increase for the continuous runs (Sephadex packing) are comparable to the values obtained from the batch runs. For the batch column, peak resolution dictates maximum sample size (in this case, this is about 9 mL). Based on the retention volumes for a given mobile-phase flow rate and the batch time for complete elution, it is possible to calculate the maximum throughput through the column. The maximum output through the continuous unit is a function of the sample feed-injection rate, mobile-phase flow rate, and column pressure. The continuous unit may be considered as a series of batch units. If it is possible to obtain the same retention volume (as the batch unit) for the same packing height and type of packing, then the throughput of the continuous unit is clearly much higher than the batch unit. In this case, the retention volume through the continuous unit was not the same as the batch unit. For example, in the case of the batch column (0.267 m packing height, 6 mL/min mobile-phase flow rate, and 6 mL injection), the retention volume for the lipase peak was 78 mL (see Fig. 3). The time taken for

the sample to completely elute out of the column was 50 min. In the case of the continuous run with Sephadex (packing height 0.24 m, mobile-phase flow rate 28 mL/min, and sample feed rate 0.5 mL/min), the retention volume for the lipase peak was about 6 mL. In spite of this, the amount of sample processed per hour is 30 mL for the continuous unit, compared to about 7.2 mL for the batch unit. Increasing the retention volume in the continuous unit will clearly result in throughputs that are much larger, and this is a function of column pressure, number of outlets in the unit, and so on.

SUMMARY AND CONCLUSIONS

Crude porcine lipase was purified by size-exclusion chromatography, using two different types of packings in both a batch and a continuous unit. The batch studies were useful in designing the continuous rotating unit. The percentage activity recovered in all cases was greater than 80%, and the x-fold increase in activity was also in double digits in all cases. An improved method of tuning the continuous chromatographic system was designed. Flow-visualization studies were conducted to optimize the continuous unit. The method utilized a dye to tag different components present in the feed, so that the separation could be visually observed. This method is effective, because it provides the operator with a visual feedback of the effects of various operating conditions on the separation process. Further purification, if desired, can be achieved by packing the unit with ion-exchange or affinity-chromatography packing.

ACKNOWLEDGMENTS

P.W.G. gratefully acknowledges fellowship from the Graduate School at University of New Hampshire. P.T.V. thanks A. Ballesteros of Instituto de Catalisis, Madrid for fruitful discussions on lipase assay techniques.

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