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Chromatographic Processing Scheme for Continuous Harvesting of rec-Prourokinase from Serum Free Medium Cell Cultures

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Abstract. Continuous perfusion cell culture, using albumin containing medium, offers the potential advantages of higher recombinant Prourokinase (r-ProUK) yields, higher initial product purity and increased throughput compared to batch culture technology using medium supplemented with fetal bovine serum. We have characterized the production of r-ProUK in medium supplemented with a lipid rich bovine serum albumin (Albumax) in a perfusion system. The results of these studies showed that it was necessary to modify the r-ProUK batch recovery scheme to process r-ProUK from a perfusion system. To accommodate large volumes of perfusate harvested over a ten to fourteen day production cycle, cation exchange and hydrophobic interaction chromatography (HIC) resins were identified that had increased product binding capacity, better flow characteristics and wider pH ranges which allowed caustic cleaning. The mobile phase composition, pH and ionic strength were modified to improve r-ProUK yields from the identified resins, and procedures were developed to eliminate r-ProUK degradation products. Strategies were defined for processing continuous harvest, which contained four to seven times the amount of r-ProUK of batch harvests.

Keywords: perfusion cell culture, hydrophobic interaction chromatography, cation exchange chromatography

Introduction

Recombinant Prourokinase (r-ProUK) is a form of the naturally occurring plasma protein produced by genetically engineered hybridoma cells (Lo and Gilles, 1991). This molecule consists of 411 amino acids, and contains 12 di-sulfide bonds. r-ProUK is the precursor of recombinant urokinase (r-UK), which is formed by hydrolysis of the Lys(158)-Ile(159) peptide bond (Marcotte et al., 1992). Prourokinase functions as a plasminogen activator in the fibrinolytic system. Potential therapeutic uses of r-ProUK in the general area of thrombolytics are currently being evaluated (Henkin and Haire, 1994; Lenich et al., 1992).

In the manufacture of r-ProUK, it is important to minimize the conversion to r-UK so that yields are maximized and product integrity is preserved. This is because the presence of r-UK will cleave r-ProUK to generate more undesired r-UK in an autocatalytic fashion. Similarly in vivo, plasma Prourokinase is also presumed to be stabilized by reduction of UK levels through the scavenging action of Plasminogen Activator Inhibitors-1 and -3 (Henkin and Haire, 1994). The manufacture of biological therapeutic proteins using mammalian cells traditionally has used batch culture processes with serum containing media. We have shown that the use of perfusion cultures can triple the amount of r-ProUK produced over batch cultures (unpublished results). In order to take full advantage of increased product yield, the isolation process used for batch processing had to be modified to accommodate increased product formation and to minimize product

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breakdown. This report focuses on modifications to the isolation process that were required to convert from a batch to a perfusion operation.

Experimental

1. *Cell Culture.* The hybridoma cell line SDU 4.1-9 containing the gene for r-ProUK production was serially propagated in modified William's medium containing heat inactivated (56°C/30 min) fetal bovine serum (final concentration 5%). For r-ProUK production the cell suspension was diluted 1 : 10 and transferred to hybridoma serum free medium (HSFM; GIBCO BRL/Life Technologies, Inc., Gaithersburg, MD) containing 30 KIU/ml aprotinin and 0.15 to 0.25% Albumax (GIBCO) that was heat treated at 65°C for 75 min. r-ProUK was produced over a seven day growth period at 37°C in batch cultures or for nine to twenty-one days in perfusion studies.
2. *Cation Exchange Chromatography.* SP-Sepharose® FF (fast flow) was tested to determine its r-ProUK binding capacity. Studies were conducted using 0.9 cm diameter columns (Spectrum Medical Industries, Inc. Los Angeles, CA) with 4 cm bed heights. The superficial velocity through these columns was approximately 200 cm/h. The columns were cleaned with ten bed volumes of 0.1 N sodium hydroxide followed by ten bed volumes of water for injection (WFI). The columns were equilibrated with twenty bed volumes of 10 mM MES (2-[*N*-Morpholino] ethanesulfonic acid; Sigma Chemical Co., St. Louis, MO) adjusted to pH 5.6 and washed after the load with ten bed volumes of the same solution.

   Loading studies on SP-Sepharose of perfusion culture harvest were carried out at laboratory scale as summarized in Table 1. For these studies all columns were loaded with r-ProUK at 2.4×10^5 Relative Units (RU) of r-ProUK per liter of resin. Chromatographies were conducted at 2 to 8°C. Columns were cleaned with ten bed volumes of WFI followed by five bed volumes of 0.5 M sodium hydroxide and another 10 bed volumes of WFI. Columns were equilibrated with ten bed volumes of the 10 mM MES described above. After loading, the columns were washed with 10 bed volumes of 10 mM MES and 10 bed volumes of 0.1 M sodium chloride/10 mM PIPES/pH 7.0. The r-ProUK was eluted with 0.3 M sodium chloride/10 mM PIPES/ pH 7.0. After elution the remaining protein was stripped from the column with ten bed volumes of 1 M sodium chloride/10 mM EPPS (*N*-[2-Hydroxyethyl] piperazine-*N*′-[3-propanesulfonic acid]; Sigma Chemical Co., St. Louis, MO)/pH 8.0 followed by a repeat of the cleaning cycle.
3. *Hydrophobic Interaction Chromatography.* Bio-Rad Methyl (Bio-Rad Laboratories, Hercules, CA), Toyopearl Butyl, Toyopearl Phenyl (TosoHaas, Montgomeryville, PA), and Phenyl Sepharose (low sub) (from Pharmacia Biotech, Piscataway, NJ) were tested for r-ProUK binding. For these evaluations the r-ProUK load was adjusted to 1.0 M ammonium sulfate (by mixing with a 2.0 M solution) at pH 7.0 prior to loading. The r-ProUK was eluted with a 10 bed volume gradient from 0 to 100% B, with Buffer A = 1.0 M ammonium sulfate/10 mM ACES(2-[(2-Amino-2-oxoethyl)amino] ethanesulfonic acid; Sigma Chemical Co., St. Louis, MO)/pH 7.0 and Buffer B = 20 mM citric acid-NaOH/50 mM sodium chloride/pH 4.5.

Table 1. Comparison of conditions for SP-Sepharose® fast flow chromatographies of perfusion culture harvests and batch culture harvests.

	Perfusion recovery		Batch recovery
Load time	10 days	3 days	5 hours
Superficial velocity (cm/h)	6	18	375
Column diameter (cm)	2.5	1.5	1.5
Bed height (cm)	7.5	7.5	7.5

Additional studies with Phenyl Sepharose were conducted with 0.9 cm diameter columns with 4 cm bed heights. These columns were run at approximately 40 cm/h. The resin was prepared by cleaning with three bed volumes of 0.5 M sodium hydroxide followed by six bed volumes of WFI. The columns were equilibrated with eight bed volumes of 1.0 M ammonium sulfate buffered with 0.1 M phosphate.

Results and Discussion

1. *Cell Culture.* The results (Table 2) of perfusion culture studies using SDU 4.1-9 cells grown in HSFM with 0.25% Albumax showed two primary advantages: 1) the culture could be maintained for an

Table 2. Summary of perfusion cell culture development studies.

Study number	Length of culture (hours)	Ratio of r-ProUK produced (perfusion : batch)	Ratio of medium consumed (perfusion : batch)	Maximum harvest % (r-UK)
1	210	3.39	2.62	17.9
2	320	4.59	4.00	ND
3	496	3.19	2.99	ND
Perfusion culture average	342	3.72	3.20	
Batch culture average	190	1.00	1.00	1.30

Cells were grown in medium containing 0.2% albumin for batch culture studies and 0.25% Albumax in perfusion studies. Cells produced 3.72 times the amount of product compared to batch cultures. If production was normalized on a volume to volume basis, the increase was 17%. ND = not determined.

Table 3. Comparison of technical specifications of several cation exchange resins as shown in the vendor's literature.

	Current	SP-Sepharose® fast flow
Total ionic capacity (mmol/ml)	NA	0.18–0.25
Nominal particle size (μm)	40	90
Bead structure	Silica based	Cross linked agarose, 6%
Operational pH	4.5–10	4–13
Cleaning pH	2–10	3–14
Normal linear flow rate (cm/h)	400–1200	400–700
Capacity (mg/ml resin)	50–67	120
	Unspecified protein	Bovine serum albumin

NA = not available.

extended period (an average of 342 h compared to 190 h for batch cultures) and thereby produced more product and 2) cells produced an average 2.07 times more r-ProUK when compared to batch culture and normalized to 190 h (17% more product on a volume to volume basis.)

2. *Recovery Process.* The typical recovery process for r-ProUK includes a cation exchange chromatographic capture step followed by hydrophobic interaction chromatography. After the first two steps, other purification procedures involving membrane filtration, gel permeation and/or affinity chromatography are employed to prepare final purified bulk r-ProUK. This study is focused on the first two steps for processing the continuous harvest which was projected to contain approximately four times the amount of r-ProUK of a batch harvest.

Cation Exchange Chromatography

For the capture step SP-Sepharose FF was evaluated. This resin was selected based on its specifications which are summarized in Table 3. The key factors for its selection were: wide pH range (which would allow caustic cleaning), good flow characteristics (predicted by its larger particle size) and high capacity (as predicted by marker proteins such as bovine serum albumin and lysozyme). This resin was evaluated for its r-ProUK binding capacity (Fig. 1). For these evaluations, the dynamic capacity was defined as the amount of r-ProUK bound to the resin when the total amount of r-ProUK in the effluent fraction equaled five percent of the activity loaded. The results of these r-ProUK binding capacity determinations at ambient temperature (18–22°C) and 2–8°C are summarized in Table 4.

Table 4. Cation exchange capacity study summary.

Resin	Temperature	Dynamic capacity 10^6 RU/L	Total capacity 10^6 RU/L
Current silica based	Ambient	17	ND
SP-Seph. FF	Ambient	38	54
SP-Seph. FF	2–8°C	54	68

ND = not determined.
RU = Prourokinase activity by S2444 assay in relative units.

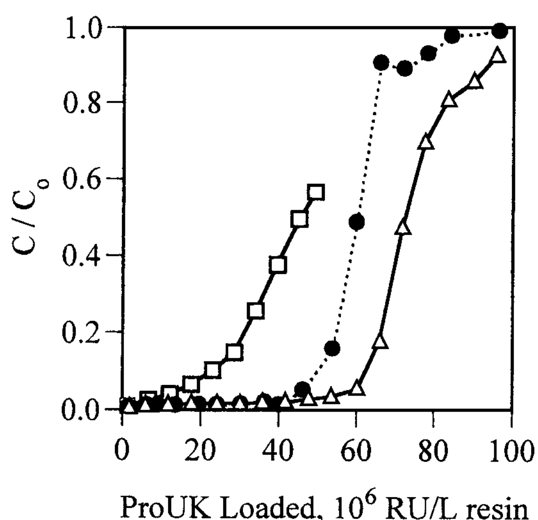

Figure 1. Breakthrough curves of ProUK in filtered cell culture harvest on cation exchange chromatography, determined as the ratio of effluent to load concentration (C/C_0) at a superficial velocity of 200 cm/h, measured at the following conditions: current resin at 20°C (□), SP Sepharose at 20°C (●), and SP Sepharose at 4°C (△).

SP-Sepharose FF was chosen for further development because of its increased r-ProUK capacity compared to the current resin. The buffer composition, pH and ionic strength were modified to optimize percent recovery and purity of r-ProUK. This resin was found to have approximately twice the capacity of the current silica based resin. This can probably be attributed to the strong sulfonated functionality on the SP-Sepharose surface as compared to the relatively weak carboxylic acid ligands of the current silica based resin. This further confirms that SP-Sepharose resin has higher protein binding capacity (Table 4) which is also a function of ligand density and pore size distribution. Since the Stoke's radius for r-ProUK is estimated around 30–35 Å, the active binding sites inside the macropores of this resin (≥300 Å) should be accessible to r-ProUK molecules.

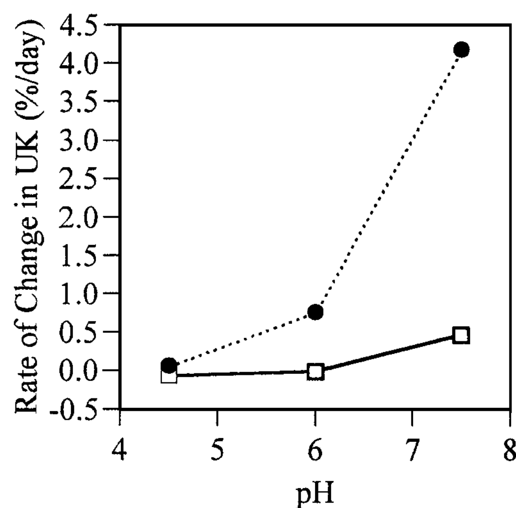

Figure 2. Stability profile for ProUK cation exchange eluate as a function of pH, measured at the following conditions: 2 to 8°C (□), and ambient [20°C] (●).

Several modifications were made in the early recovery steps to maximize the yield and quality of r-ProUK from perfusion cultures. For example the perfusate was maintained at 2–8°C in the holding tank between the bioreactor and the cation exchange column. The pH was continuously adjusted to pH 5.6, which was optimum for efficient binding during the cation exchange step, and reduced the rate of r-UK formation (Fig. 2). In order to accommodate longer loading and elution time for perfusion cell cultures, it is necessary to lower the operating temperature to 2–8°C for cation exchange chromatography so that the r-ProUK stability can be preserved. It appeared that temperature had a significant impact on dynamic capacity of the cation exchanger when it was loaded with filtered cell culture harvest. This was confirmed in a second study (Fig. 3) which used a common pool of harvest to load columns at three different temperatures. The observation that capacity increased at lower temperature appears to be related to the culture medium, because studies with r-ProUK suspended in phosphate buffered saline (Fig. 4) show increasing capacity with increasing temperature.

Hydrophobic Interaction Chromatography (HIC)

BioRad Methyl, Toyopearl Butyl, Toyopearl Phenyl, and Phenyl Sepharose (low sub) were chosen for evaluation based on their specifications as detailed in Table 5. The key factors for their selection were: wide

Table 5. Comparison of technical specifications of several hydrophobic interaction chromatography resins.

	Current	BioRad Methyl	Toyopearl Butyl	Toyopearl Phenyl	Phenyl Sepharose
Bead structure	Silica based	Methacrylic copolymer			Cross-linked agarose, 6%
Nominal particle size (μm)	40	50	35	35	90
Working pH stability	4.5–10		2–12	2–12	3–13
Cleaning pH stability	2–10	2–14	2–14	2–14	2–14
Albumin capacity (mg/ml resin)		25	35	28	24

Table 6. Hydrophobic interaction chromatography screening study summary.

Resin	Activity recovery (%)	Protein recovery (%)	Protein balance (%)	Relative specific activity
Current silica based	87.7	93.9	96.0	1.00
BioRad Methyl	75.2	88.6	91.3	1.04
Toyopearl Butyl	12.9	19.9	23.9	0.80
Toyopearl Phenyl	74.7	86.1	93.7	0.98
Phenyl Sepharose (low sub)	81.1	93.6	102.4	0.98

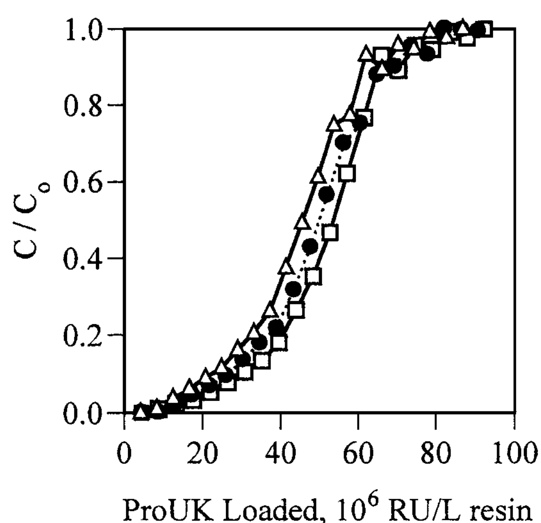

Figure 3. Breakthrough curves of ProUK in filtered cell culture harvest on SP Sepharose® Fast Flow, determined as the ratio of effluent to load concentration (C/C_0) at a superficial velocity of 200 cm/h of 4°C, measured at the following temperatures: 4°C (□), 12°C (●), and 20°C (△).

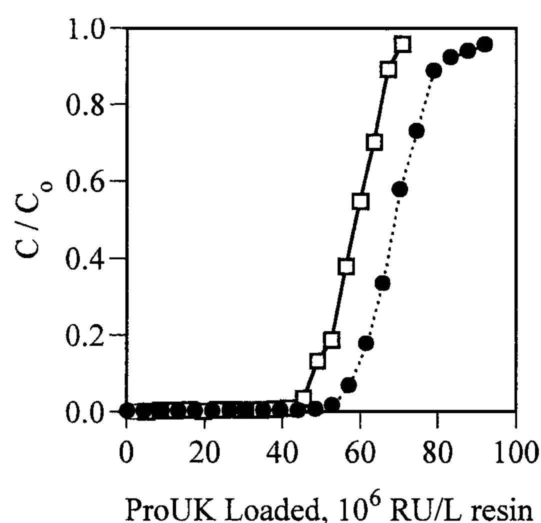

Figure 4. Breakthrough curves of ProUK in phosphate buffered saline on SP Sepharose® Fast Flow, determined as the ratio of effluent to load concentration (C/C_0) at a superficial velocity of 200 cm/h of 4°C, measured at the following temperatures: 4°C (□), and 20°C (●).

pH range (which would allow caustic cleaning), good flow characteristics (predicted by their larger particle size) and high capacity (as predicted by their albumin binding capacity). The r-ProUK activity and protein recovery of these four HIC resins were compared to the current silica based resin (Table 6). The activity recovery (81.1%) for Phenyl Sepharose was closest to that of the silica based resin (87.7%), which has a propyl

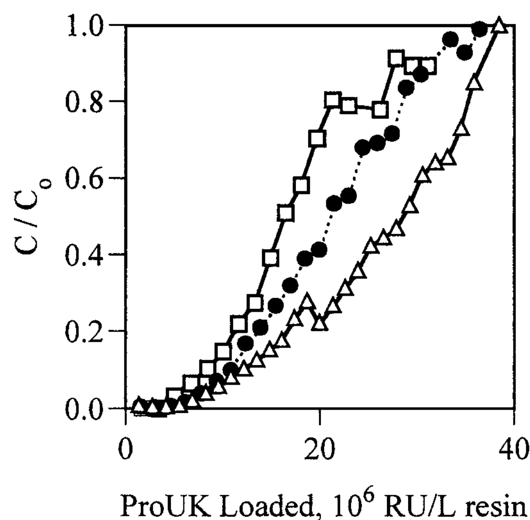

Figure 5. Breakthrough curves of ProUK in a 1.0 M ammonium sulfate solution on Phenyl Sepharose® Fast Flow low sub, determined as the ratio of effluent to load concentration (C/C_0) at a superficial velocity of 43 cm/h and temperature of 4°C, measured at the following pH's: 6.0 (□), 7.0 (●), and 8.0 (△).

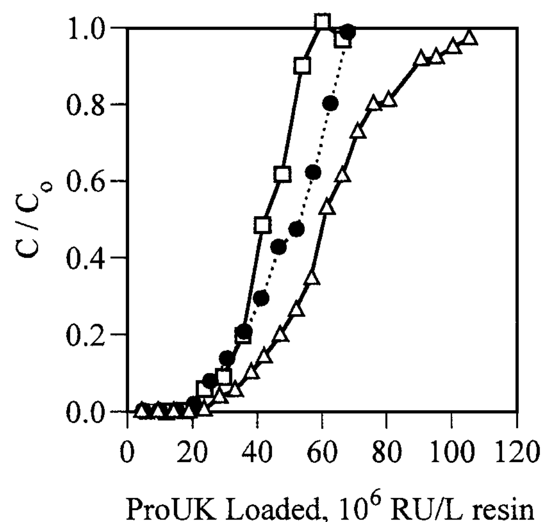

Figure 6. Breakthrough curves of ProUK in a 1.0 M ammonium sulfate solution on Phenyl Sepharose® Fast Flow low sub, determined as the ratio of effluent to load concentration (C/C_0) at a superficial velocity of 43 cm/h and temperature of 20°C, measured at the following pH's: 6.0 (□), 7.0 (●), and 8.0 (△).

ligand. The r-ProUK peaks from the phenyl resin eluted shortly before the peak for the silica based resin. The butyl HIC resin was rejected due to its poor activity recovery and the low retention time of the peak from the methyl resin suggested that its performance would be much different than that of the silica based resin. From the remaining resins Phenyl Sepharose (low sub) was chosen for further study.

In order to further define the operating conditions for Phenyl Sepharose (low sub), loading studies were performed at different temperatures and pH's. Figure 5 shows the breakthrough curves for r-ProUK on Phenyl Sepharose at 4°C. The capacity of the resin is increasing with pH, and all the pH's evaluated were below the isoelectric point of r-ProUK. Several other authors have reported changes in selectivity in hydrophobic interaction chromatography with changes in pH (Heinitz et al., 1988 and Fausnaugh et al., 1984). Although most of the cases cited show decreased retention with increasing pH, there were several examples which showed increased retention like that observed with r-ProUK. As noted by Heinitz et al., there are no general relationships between p*I* and retention behavior in hydrophobic interaction chromatography. Figure 6 shows similar results when the studies were run at 20°C with a more concentrated load sample.

The effect of flow rate was evaluated by repeating the pH 7 and ambient temperature condition at various superficial velocities (Fig. 7). As expected the r-ProUK breaks through sooner as the flow rate is increased. Since we observed that the dispersion for these binding studies was more pronounced at lower temperature, the lower dynamic capacity may simply be a reflection of slow kinetic processes for r-ProUK molecules to

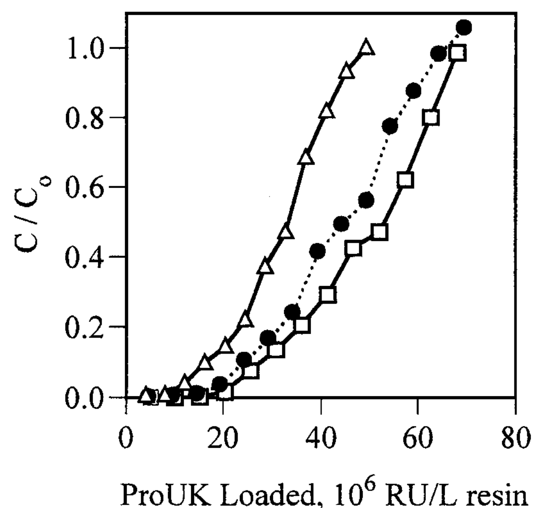

Figure 7. Breakthrough curves of ProUK in a 1.0 M ammonium sulfate solution on Phenyl Sepharose® Fast Flow low sub, determined as the ratio of effluent to load concentration (C/C_0) at a pH of 7.0 and temperature of 20°C, measured at the following superficial velocities: 43 cm/h (□), 86 cm/h (●), and 174 cm/h (△).

penetrate and absorb onto the active phenyl ligands inside the Sepharose microporous surfaces. Furthermore, because the r-ProUK stability increases at lower temperature (2–8°C vs. ambient) as shown in Fig. 2, it becomes necessary to maintain 2–8°C during the gradient elution of this Phenyl-Sepharose column. Since the dynamic capacity usually defines the column size required for this separation, it would be more appropriate to load the product at even slower flow rate to minimize the mass transfer effects.

Perfusion Recovery Schemes

The main options to consider for recovery from this perfusion cultures were:

1. Storage of the r-ProUK activity in a perfusate (harvest) hold tank versus holding the activity on the first column for many days.
2. The use of single versus multiple column runs.

The option of storing the harvest in hold tanks was undesirable because of the requirement for additional capital expense involved in storing large volumes from the continuous cell culture process. Therefore it was important to show the feasibility of holding the r-ProUK activity on the cation exchange capture step for ten to fourteen day production cycle of the perfusion system. Technically it was shown to be possible to load a lab scale column continuously for ten days and still achieve a 95.3 percent r-ProUK activity recovery (Table 7). The next option to consider was whether to scale the chromatography columns to recover the continuous harvest in one cycle with large columns or in multiple cycles with smaller columns. These options are illustrated in Fig. 8, which shows the scale of the recovery train if the laboratory process was increased to a 1000 l bioreactor. This figure shows the further process option of storing the eluates from the multiple cycle process so they can be pooled to perform later recovery steps in a single cycle. No significant differences in r-ProUK yield or quality were observed when the r-ProUK was loaded continuously for ten days, compared to multiple loads of three to four days (Table 7). When assessing the advantages of the single versus the multiple cycle process, cost is the next factor to consider. Over the useful life of the chromatography resins there should be no difference in material cost between the options. The equipment cost for the multiple cycle process should be less in this specific case because the equipment from the existing recovery process (for batch cell culture) could be used. Either of the perfusion recovery schemes would require constant monitoring by production personnel. Therefore the headcount requirement for the two perfusion schemes would be the equivalent. Another factor to consider is contamination risk or a mechanical failure during the course of a continuous culture process. This would result in the loss of an entire batch in the single cycle process but possibly only a partial loss in the multiple cycle option. The use of the multiple cycle option could allow the use of existing equipment and facilities while taking advantage of the increased productivity of the perfusion culture technology.

Table 7. SP-Sepharose loading study summary.

Condition load time	Control 5 hours	Perfusion 3 days	Perfusion 10 days
Activity recovery	96.9%	98.2%	95.3%
HPLC ProUK purity	81.0%	82.5%	81.6%
UK activity	3.0%	2.7%	3.9%

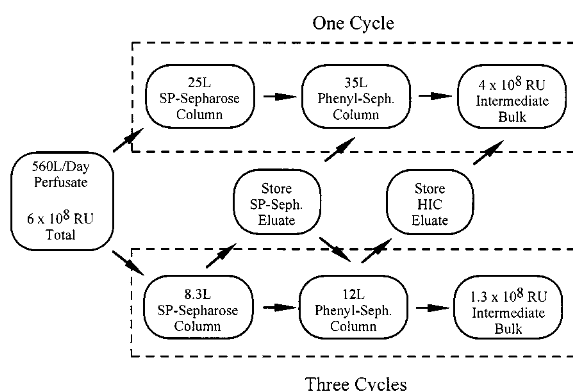

Figure 8. Perfusion cell culture recovery options.

Conclusions

During the development of a new r-ProUK from perfusion culture system with a serum free medium it was necessary to develop new resins for the first two steps in the recovery in order make use of the existing recovery facilities. A strong cation exchange resin (SP-Sepharose®) was chosen for the capture step and Phenyl Sepharose® (low sub) was identified for the

hydrophobic interaction chromatography step. All of these resins are not silica based and thus offer the advantage of caustic cleaning. Similar r-ProUK yield and quality were observed when the r-ProUK was loaded continuously for ten days, compared to multiple loads of three to four days. The multiple load option was considered the best choice for this product, because capital expense could be avoided by the use of existing equipment.

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