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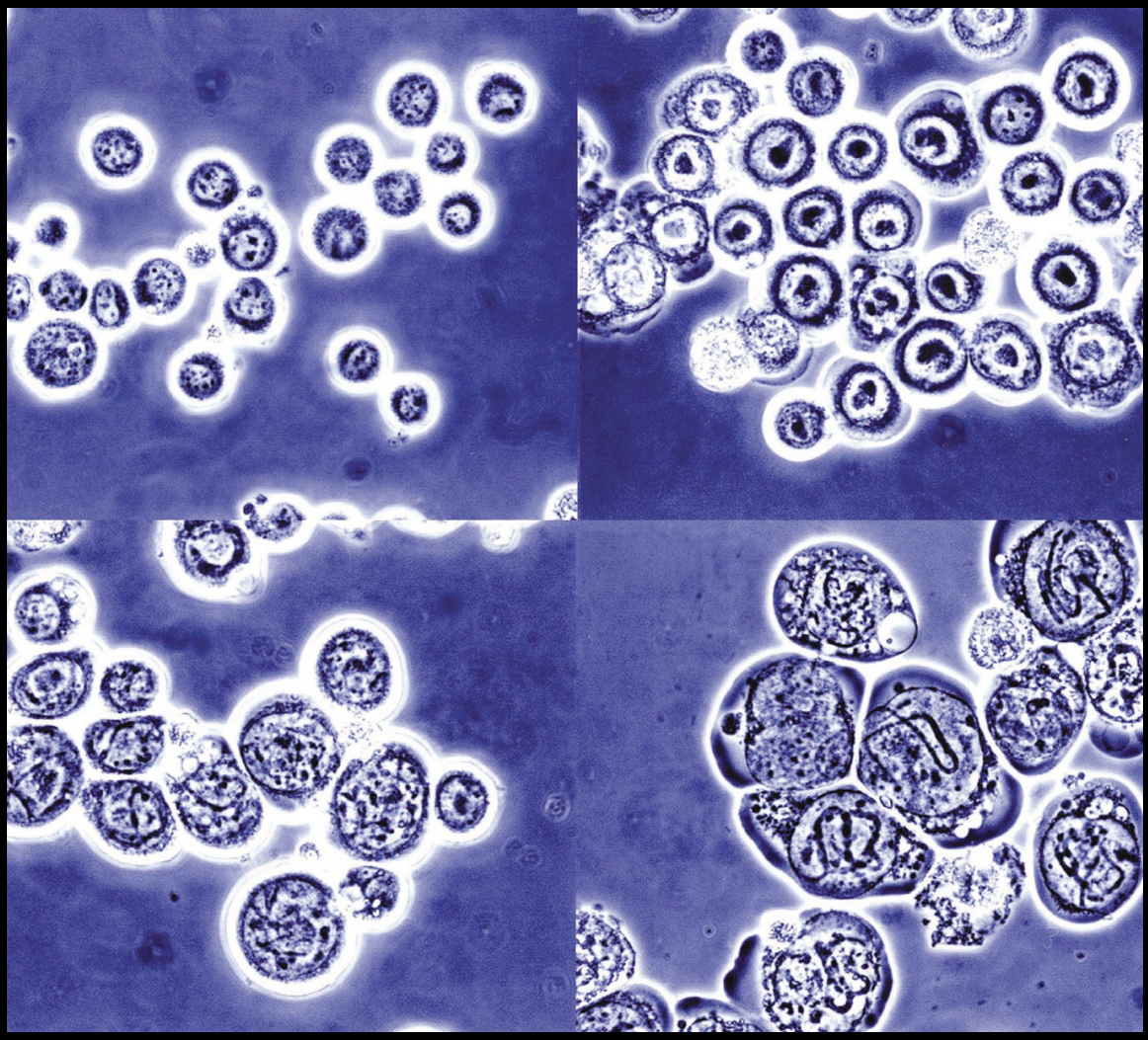

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A Rapid Method for the Capture and Purification of a Protease Sensitive Protein

BY CHRISTOPHER W. KEMP

Portions of this article were presented at the WilBio-2002 Conference on Baculovirus and Insect Cell Culture.

Protein p75 is a 75 kDa vaccine candidate that is produced as a secreted product from insect cells infected with a recombinant baculovirus. Small-scale (1L) studies were conducted to establish optimal conditions for the expression and purification of p75, and a protocol was developed using three ion-exchange steps followed by size-exclusion chromatography.

The final purified yield of the p75 protein was 10 mg/L with a purity level of greater than 95%. Increasing the process size to the 40L scale resulted in the production of full-length p75 protein, yet the material degraded prior to elution from the initial ion-exchange column. The degradation of the protein appeared to be related to the increased amount of time required to process the larger batch size.

The degradation was reduced by the addition of ion-exchange resin, directly to the bioreactor tank, at the time of virus addition. This in-situ method of product capture reduced the time for the initial ion-exchange step from a minimum of six hours to less than one hour, and allowed for the successful scale-up of p75 production.

Introduction

Kemp Biotechnologies, Inc. is a contract service organization that produces and purifies proteins. The baculovirus expression vector system (BEVS) is one method utilized in the facility for the production of recombinant proteins, and typically works without significant difficulties. However, some proteins are produced in insoluble forms, and degradation can occur. This article will focus on this degradation issue, and present a method to stabilize a protease-sensitive protein that has been produced at the 40-liter scale.

The BEVS naturally supports high levels of intrinsic protease activity. In nature, the infection cycle results in the complete degradation of the insect larvae, and Sf9 cells infected in-vitro with recombinant baculovirus particles lyse within a week. There is no definitive review of the protease types associated with the BEVS system. However, it has been reported that the *Autographa californica* M nuclear polyhedrosis virus (AcMNPV) genome contains a papain-like sequence,[1] and produces a cathepsin L-like protease.[2]

It has been our experience that approximately 20% of the proteins we express with BEVS demonstrate some degree of degradation during a normal expression cycle (72 hours post-infection). Typically, we will observe the formation of a truncated protein form. This may be controlled by adjusting the multiplicity of infection (MOI) or time of harvest, as well as changing the host cell line. The formation of truncated protein forms has also been reported in the literature.[3] In this case, the truncation produced a soluble 62 kDa product from an insoluble 71 kDa putative capsid protein of the hepatitis E virus.

Degradation of the protein of interest (p75) was not detected until the process was increased in scale from the one-liter to the forty-liter level. This degradation was associated with the ion-exchange capture step where the protein eluted as a degraded product. In this article, we will present a method for the capture of secreted proteins as they are produced in the bioreactor. The method is dependent on the pI of the secreted protein and the type of chromatography resin employed. The materials and conditions used in this report are specific for the p75 protein, and may not be optimal for other proteins. Optimal conditions must be determined for each protein of interest. Prior to their use in bioreactor cultures, we routinely screen a variety of resins at the shake-flask level.

Materials and Methods

Baculovirus Expression

The gene encoding p75 was cloned into the pVL1392 baculovirus expression vector (Pharmingen) and then cotransfected with Baculogold linearized baculoviral DNA into *Spodoptera frugiperda* Sf9 cells using methods described in the Baculovirus Expression Vector System Manual published by Pharmingen.[4] The virus was cloned through two rounds of plaque purification, where clone selection was based on product expression, as determined by Western Blot analysis using a p75-specific monoclonal antibody. The selected clone was amplified, and the optimal conditions for expression (MOI / time course) were determined using shake-flask cultures.

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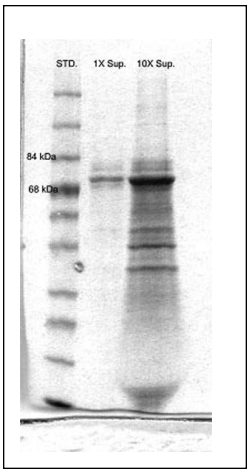

Figure 1. Full-length p75 protein in unconcentrated and concentrated culture supernatant. SDS PAGE 4-20% Tris-Glycine gel run under reducing conditions and developed using Coomassie Blue stain. Note the absence of a major degradation band.

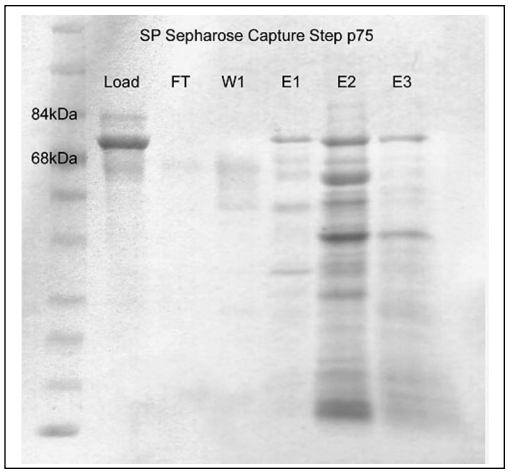

Figure 2. P75 degradation observed during the initial capture step of a 40-liter production run. Note the integrity of the protein in the load sample (concentrated supernatant), and the degradation evident in eluate fraction 2 (E2). SDS PAGE 4-20% Tris-Glycine gel run under reducing conditions and developed using Coomassie Blue stain.

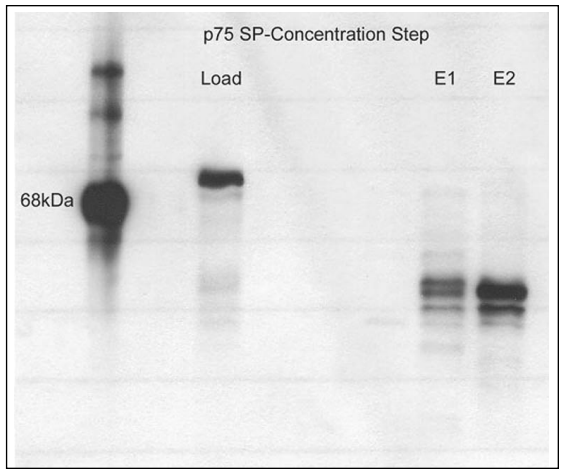

Figure 3. P75 degradation observed during the initial capture step of a 40-liter production run. SDS PAGE 4-20% Tris-Glycine gel run under reducing conditions transferred to nitrocellulose and probed using a p75 specific monoclonal antibody.

All of the p75 protein expression experiments were conducted with the Sf21 cell line cultivated in Sf900-II medium (Invitrogen Cat.#10902-070). The p75 protein was secreted into the culture medium and was detected using 4-20% SDS-PAGE gels and Western blot analysis. The optimal conditions for expression of p75 were determined to be at an MOI of 5 with a harvest time of 72 hours, post-infection. The cells were inoculated at a density of $2.5x10^5$ cells per ml and virus was added to the culture when the cell density reached $2x10^6$ cells per ml. The bioreactor culture conditions included a temperature of 27°C, a dissolved oxygen level of 50%, and an agitation rate of 80 rpm. The pH of the culture was monitored, but not controlled, and it was found to not change by more than 0.2 pH units throughout the culture.

Seventy-two hours after the addition of the virus, the culture supernatant was isolated using centrifugation at 3000 x g for 10 min. The cell pellet was discarded, the culture supernatant was concentrated 10-fold using a 10,000 MWCO hollow-fiber ultrafilter (AG Technology Cat.#UFP-10-C-9A), and then held on ice, or at -80° C, prior to purification.

Original Purification Scheme

All procedures were conducted at 2-8° C, unless otherwise noted, and are scaled for a 40-liter expression. Clarified supernatant was diluted with two parts of deionized water prior to adjusting the pH to 4.5 with 1 N hydrochloric acid. The material was stirred at room temperature for 15 minutes, and clarified with centrifugation at 10,000 x g for 20 min at 4°C. The supernatant was collected and filtered through a 0.45 µm bottle-top filter (Schleicher and Schuell, Cat#67240).

The clarified material was loaded onto 300 ml of SP Sepharose Fast Flow resin (Pharmacia Biotech, Cat.#17-0729), and then equilibrated with 25mM sodium acetate buffer (pH 4.5 and containing 100 mM sodium chloride) using a rate of 150 cm/h. The column was washed to baseline with the load buffer, and the p75 protein was eluted using the same buffer, but with a final sodium chloride concentration of 500 mM. The eluate pool was analyzed using SDS-PAGE for the presence of the p75 protein, and dialyzed against 50 volumes of 25 mM Tris at pH 8.0. The dialyzed material was loaded at 125 cm/h onto 50ml of Q Sepharose Fast Flow resin (Pharmacia Biotech, Cat.#17-0510), and then equilibrated with 25 mM Tris, pH 8.0, while connected in series to 50ml of SP Sepharose Fast Flow resin.

After 10 column volumes (50 ml) of buffer had been washed through the columns, the Q Sepharose column was disconnected and the p75 protein was eluted from the SP Sepharose column with the load buffer (final sodium chloride concentration of 100 mM). The eluate fractions were examined for the presence of p75 using SDS-PAGE, pooled and loaded onto a HiPrep 16/60 Sephacryl S-100 HR column (Pharmacia Biotech, Cat.#17-1165-01), and then equilibrated with 25 mM Tris-HCl (pH 8.0 containing 100 mM NaCl) at a flow rate of 0.8 ml per minute. The eluate fractions were examined for p75 using SDS PAGE and Western Blot analysis, and then pooled, concentrated, and stored at -80°C.

In Situ Capture Method

An aliquot of SP Sepharose Fast Flow resin (7.5 ml resin slurry per liter culture volume) was aseptically removed from a previously unopened bottle and added directly to the bioreactor vessel at the time of virus addition. Alternatively, the resin may be washed 2X with dionized water, and then resuspended into one volume of deionized water. The washed resin may then be sterilized in an autoclave at 120°C for 30 min prior to being added to the culture vessel.

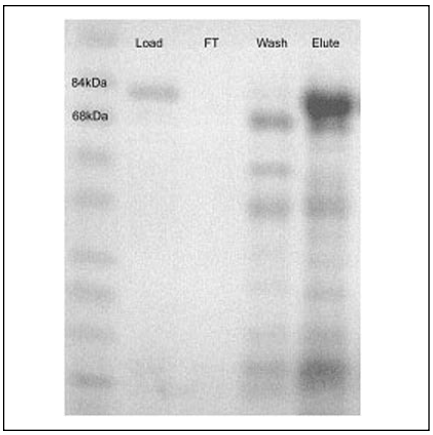

Figure 4. Capture and elution of p75 from culture supernatant using SP Sepharose. Resin was added directly to culture supernatant collected from a test expression of the p75 protein. The resin was washed using BisTris buffer as described in the Materials and Methods section, and eluted with SDS PAGE sample buffer. SDS PAGE 4-20% Tris-Glycine gel run under reducing conditions and developed using Coomassie Blue stain.

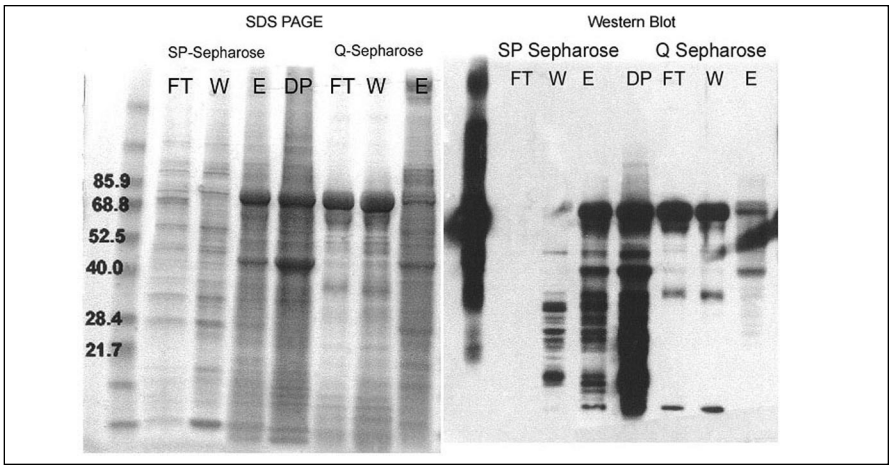

Figure 5. SDS PAGE and Western Blot analysis illustrating the purification of p74 protein using the in-situ capture method. Note the presence of the p75 degradation product in the eluate (E) of the SP column, and from the dialysate pellet (DP) from the buffer exchange prior to the Q column. The DP sample is highly concentrated relative to the other fractions. Also evident is the removal of the p75 degradation product in the Q Sepharose flow-through column step. SDS PAGE 4-20% Tris-Glycine gel was run under reducing conditions and developed using Coomassie Blue stain. Western blot analysis used a p75 specific monoclonal antibody to develop the image of the immunoreactive proteins transferred to a nitrocellulose membrane from a 4-20% Tris-Glycine SDS PAGE gel.

The resin was collected 72 hours post-infection by transferring the whole culture through a sieve of 70 µm nylon mesh (Fisher Scientific, Cat#08-670-199). The resin was washed from the nylon mesh into a beaker using culture medium, and then transferred to a chromatography column and washed with ten bed volumes of 10 mM BisTris, pH 6.2. The protein elution from the column was monitored at 280 nm, and was effected using the wash buffer containing 300 mM NaCl. As described above, the elution pool was analyzed, prior to dialysis, for the presence of p75 using SDS-PAGE.

All subsequent purification steps were as described in the preceding section, with the exception that the final buffer was 50 mM Tris-HCl, pH 8.0, with 100 mM NaCl, and a HiPrep 16/60 Sephacryl S-200 HR column (Pharmacia Biotech, Cat.#17-1069-01) was substituted for the S-100 column.

Results and Discussion

The expression and purification of the p75 protein yielded full-length material at the 1-liter expression scale (Figure 1). An attempt to increase the scale to 40-liters resulted in expression of full-length protein that eluted as a degraded product from the initial SP Sepharose capture column (Figures 2 and 3).

Numerous attempts were made to limit the degradation, including the addition of 1 mM PMSF (final concentration) to the culture supernatant or the concentrated supernatant, and the addition of the protease inhibitor cocktail, Complete (Roche Applied Science Cat.#1-873-580), to the concentrated supernatant fluid and column buffer. No decrease in degradation was observed using PMSF or Complete alone, or in combination (data not shown).

Our timeline for the project did not allow for an exhaustive study of protease inhibitors, therefore, we elected to investigate a possible inhibition of the protease activity through a manipulation of the abiotic environment. An attempt was made to limit the degradation by altering the pH of the initial step, so a titration experiment was performed from pH 4.5 to pH 6.5 (data not shown). It was observed that the p75 protein bound to the SP Sepharose resin at pH 6.5, with a load buffer conductivity of 6 mS/cm. Running a 40-liter batch of supernatant through the SP Sepharose column, at pH 6.5, did not alter the degradation of the protein.

An analysis of the culture medium conductivity revealed a value of 8.8 mS/cm with a pH of 6.2. A shake-flask culture (50 ml culture volume) was infected with the p75 virus, and 0.5 ml of SP Sepharose resin was added to the culture at the same time. The resin was isolated 72 hours after the virus addition, and the results are shown in Figure 4. The resin was washed using 10 mM BisTris at pH 6.2. The protein was eluted using SDS-PAGE load buffer and the results show (Figure 4) that the p75 protein was eluted as an intact molecule.

Our next step was to test the capture method at the 40-liter scale. A 40-liter bioreactor culture was initiated using Sf21 cells in SF900-II medium. The p75 virus was added at an MOI of 5 when the cell density reached $2x10^6$ cells per ml. In addition, 300 ml of SP Sepharose was added at the same time. The resin was captured onto nylon mesh resin after the 72 hour incubation at 27°C, and full-length protein was eluted from the resin along with some degraded product. The total process time for resin collection from the bioreactor, through protein elution from the resin, was 45 minutes.

The results of the first two chromatograph steps (SP capture and Q flow-through) are shown using SDS-PAGE and Western blot analysis in Figure 5. The p75 protein appears to

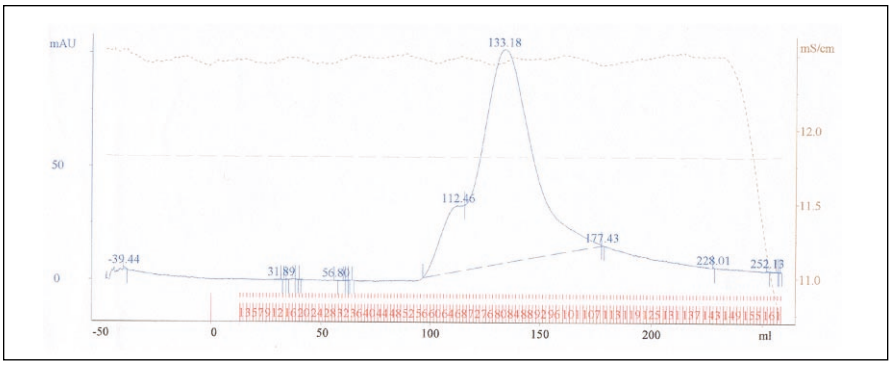

Figure 6. Chromatogram illustrating the biphasic elution peak of p75 when processed using the S-200 gel filtration column. The leading shoulder represented an immunoreactive protein of slightly lower molecular weight than the p75 protein. The degradation product was partially separated from the full-length molecule using this column step.

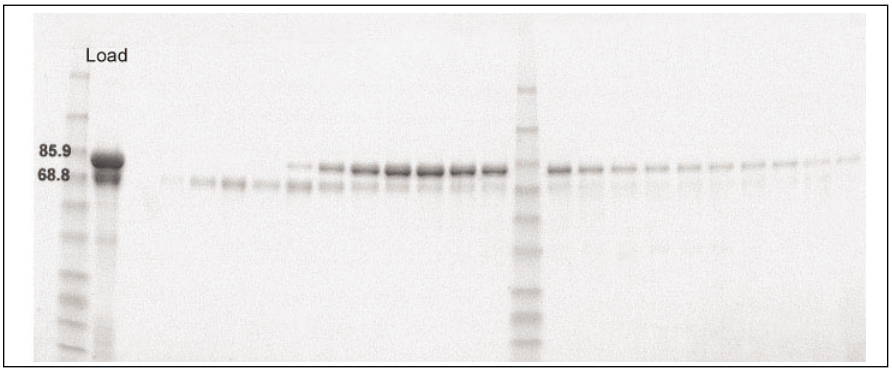

Figure 7. Elution profile of the p75 protein from the S-200 column. The lower molecular weight band represents the leading shoulder in Figure 6 and is immunoreactive against the p75 monoclonal antibody. The S-200 column partially resolved the degradation band from the full-length molecule.

be absent from the SP column flow-through, indicating that the conditions allowed a complete capture of the protein in-situ. There was a small amount of p75 protein present in the SP column, but the majority of the protein eluted with the 300 mM NaCl wash. The lane labeled DP represents the precipitate isolated from the dialysis step prior to the Q column.

The material was resuspended into Tris buffer and was highly concentrated, relative to the eluate from the SP column and the flow-through of the Q column. It is interesting to note that though the p75 degradation product (approximately 42 kDa) is present in the eluate and the dialysis pellet from the SP column step, it is absent in the flow-through of the Q column but appears in the eluate of this column.

It appears that the truncation of the protein sufficiently altered the pI of the protein to allow for this separation. The flow-through from the Q column was loaded directly onto an additional SP column at pH 8.0. The p75 protein still bound to the resin at this pH. However, note that the buffer contained no NaCl and the p75 eluted in 100 mM NaCl, as opposed to 300 mM NaCl at pH 6.2. The material appears similar to the flow-through from the Q column. This can be seen in the load fraction (Figure 6), which indicates that the second SP column functions mainly as an initial concentration step before the S-200 column.

An additional degradation product appears in the flow-through from the Q column, which increases in intensity as the material is processed through the second SP column and the concentration prior to the S-200 gel filtration step. This band is immunoreactive (data not shown) and is partially separated from the intact molecule by the S-200 column procedure (Figures 6 and 7). The purity of the pooled p75 protein, as collected from the S-200 column, was determined to be 95%, using the Agilent Bioanalyzer 2000 capillary electrophoresis system, and the final purified yield of p75 protein was 11 milligrams per liter.

Conclusion

The in-situ capture of the p75 protein onto the SP Sepharose resin allowed us to obtain full-length protein from larger scale expressions. The sensitivity of the p75 protein to proteolysis forced us to try this method of capture. However, the success of the procedure has caused us to use this method with a number of secreted proteins produced with BEVS. The success of the procedure is dependent on the affinity of the protein for the resin, and we have utilized both anion and cation exchange resins for the capture step. To date, we have not attempted to use an affinity resin for in-situ capture.

Only part of this procedure's success was due to the resin addition to the bioreactor. The other element was the recovery of the resin using the nylon mesh screen. The use of the screen lowered the process time by at least one hour, and was responsible for keeping the total process time of the initial column to less than one hour. In our opinion, the success of the purification was ultimately the result of the reduction in processing time.

Acknowledgements

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