0273-2289/01/90/0125/$13.00

Affinity Purification of Secreted Alkaline Phosphatase Produced by Baculovirus Expression Vector System

FUMING ZHANG,[1] MICHAEL W. WOLFF,[1] DAVID WILLIAMS,[1] KATIE BUSCH,[1] SYBIL C. LANG,[1] DAVID W. MURHAMMER,[1] AND ROBERT J. LINHARDT*,[1,2]

[1]Department of Chemical and Biochemical Engineering, and [2]Department of Chemistry and Division of Medicinal and Natural Products Chemistry, University of Iowa, Iowa City, IA 52242, E-mail: robert-linhardt@uiowa.edu

Received June 1, 2000; Revised August 1, 2000; Accepted October 1, 2000

Abstract

Human secreted alkaline phosphatase (SEAP) was produced in a stably-transformed *Spodoptera frugiperda* Sf-9 insect cell line (Sfb4GalT) following infection with a recombinant *Autographa californica* multiple nuclear polyhedrovirus containing the SEAP gene under control of the polyhedrin promoter. An affinity chromatographic column prepared by linking 4-aminobenzylphosphonic acid to histidyl-expoxy-Sepharose was used to isolate SEAP from the cell supernatant following removal of cells and virus and 10-fold concentration through ultrafiltration. We found that the binding of SEAP on the affinity matrix follows the Langmuir isotherm model. In addition, either recycling SEAP sample through the column for 24 h or loading high SEAP concentrations resulted in a high-purity product. Some nonspecific binding of protein on the matrix occurred when low concentrations of SEAP sample were loaded. Finally, we found that SEAP binding occurs rapidly, i.e., within 30 min of adding the SEAP sample to the affinity matrix.

Index Entries: Affinity chromatography; human secreted alkaline phosphatase; baculovirus expression vector system; insect cells; recombinant protein; protein purification.

Introduction

The baculovirus expression vector system (BEVS) is widely used to produce recombinant proteins. This system has several advantages over

*Author to whom all correspondence and reprint requests should be addressed.

mammalian expression systems, including ease of use, safety, and potentially high expression levels. In addition, the host insect cells are able to conduct most of the posttranslational modifications observed in mammalian cells *(1)*. The utility of the BEVS is limited, however, by the inability of host insect cells to perform complex glycosylation of recombinant proteins *(2,3)*. This inability to perform complex glycosylation is owing to the absence or insufficient levels of the terminal glycosyltransferases required to convert N-linked side chains to complex forms *(4)*. Recent studies have demonstrated that metabolic engineering can be used to extend the glycoprotein processing capability of insect cells to produce foreign glycoproteins with glycans that more closely resemble those produced by higher eukaryotes *(5)*. These studies involved the incorporation of mammalian glycosyltransferases into the insect cell *(5,6)*.

Secreted alkaline phosphatase (SEAP), a 64-kDa protein encoded by a 1.53-kb gene *([7]*; unpublished data), provides an excellent model protein for investigating N-linked glycosylation since it contains only a single occupied N-linked glycosylation site and no occupied O-linked sites *(8)*. To analyze the structure of the SEAP glycans, it is critical that the SEAP be of high purity to ensure that the sample is not contaminated with glycans from other proteins. Affinity chromatography is a highly selective technique that provides the means to attain highly pure proteins, sometimes in a single step *(9)*. Regarding SEAP purification, an affinity column with a matrix containing 4-aminobenzylphosphonic acid linked to histidyl-expoxy-Sepharose can be used *(10,11)*. SEAP binds specifically to the phosphonic acid moiety of the affinity matrix, while other proteins pass unretarded through the column. Thus, pure SEAP can be obtained by (1) passing the solution containing SEAP and other proteins through the affinity column, (2) washing out impurities, and (3) releasing the SEAP from the affinity matrix. While this methodology results in pure SEAP, it requires several days to load the sample onto the column (Wood, H. A., personal communication).

In the present study, we report on SEAP production by infecting a stably transformed *Spodoptera frugiperda* Sf-9 cell line expressing galactosyltransferase (Sfβ4GalT) with a recombinant *Autographa californica* multiple nucleopolyhedrovirus expressing SEAP under control of the polyhedrin promoter (AcMNPV-SEAP). Following removal of cells and virus from the supernatant through centrifugation and 10-fold concentration via ultrafiltration, we investigated the effect of different operation modes and loading amounts on SEAP purification with the affinity column. These studies resulted in methodologies that can be used to significantly reduce the time required to obtain pure SEAP.

Materials and Methods

Chemicals

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Cell Line and Virus

The modified *S. frugiperda* Sf-9 insect cell line expressing galactosyltransferase (Sfβ4GalT) under control of IE1 promoter was provided by Dr. Donald Jarvis (University of Wyoming) *(6)*. Recombinant *A. californica* multiple nuclear polyhedrovirus expressing human secreted alkaline phosphatase (AcMNPV-SEAP) under control of the polyhedrin promoter was obtained from Dr. H. A. Wood (Boyce Thompson Institute for Plant Research, Ithaca, NY) *(12)*.

Cell Growth

Sf-9 cells adapted to grow in HyQ® SFX INSECT serum-free medium (HyClone, Logan, UT) were maintained in tissue culture flasks at 27°C. These cells were used to seed Erlenmeyer flasks at a cell density of ~0.5×10^6 cells/mL. Further scale-up prior to SEAP production utilized these cells (when the density reached 3–5×10^6 cells/mL) to seed larger Erlenmeyer flasks at ~0.5×10^6 cells/mL. The shaker flask cultures were grown at 27°C and rotated at 125 rpm.

Expression of SEAP with the BEVS

Sf-9 cells (400 mL in 2-L shaker flasks) cultivated under the same conditions as just described were infected at a density of ~1.0×10^6 cells/mL at a multiplicity of infection (MOI) of 5.0. At 4 h postinfection, cultures were centrifuged (500*g* for 5 min) to remove the old medium, and the infected cells were gently resuspended in an equal volume of fresh medium. The infected cultures were incubated in a shaking incubator (27°C, 125 rpm) for approx 72 h until the viability decreased to 70–80%.

Preparation of Affinity Matrix

The affinity matrix was prepared by linking 4-aminobenzylphosphonic acid to histidyl-expoxy-activated-Sepharose *(10,11,13)*. The activation of Sepharose 6B with epichlorohydrin was performed as described by Matsumoto et al. *(14)*. Briefly, washed and vacuum-dried Sepharose (200 g) was suspended in 300 mL of water and mixed with 130 mL of 2 *M* NaOH solution and 30 mL of epichlorohydrin. The suspension was maintained at 40°C for 2 h in a shaking incubator. It was then transferred to a sintered glass funnel, and the gel was washed extensively with distilled water. Next, histidine was coupled with expoxy activated Sepharose by resuspending the Sepharose in 3 L of saturated histidine solution in 50 m*M* sodium carbonate (pH adjusted to 10.5 with solid NaOH). This mixture was allowed to react in a shaker overnight at 55°C. The derivatized Sepharose was washed in a sintered glass funnel with 2 L each of 0.2 *M* sodium citrate, 0.5 *M* NaCl (pH 2.5), and 0.2 *M* Tris–0.5 *M* NaCl (pH 10.5), followed by extensive washing with distilled water.

Finally, the histidyl-Sepharose was coupled to 4-aminobenzylphosphonic acid by diazotization. Briefly, 160 mL of 0.5 *M* sodium nitrite

was reacted with 15 g of 4-aminobenzylphosphonic acid dissolved in 1.5 *M* HCl in an ice bath for 20 min. This solution was added to the cold histidine-Sepharose suspended in water. The pH was rapidly adjusted to 9.5 with cold 1.5 *M* NaOH, and the mixture was shaken at 4°C for 4 h. After completion of diazotization, the matrix was washed with 2 L each of 0.2 *M* sodium citrate, 0.5 *M* NaCl (pH 2.5), and 0.2 *M* Tris–0.5 *M* NaCl (pH 10.5), followed by extensive washing with distilled water until the eluent was clear.

Pretreatment of Insect Cell Culture Medium Containing SEAP

The medium containing SEAP was centrifuged at 500*g* for 10 min to remove insect cells. Virus was pelleted at 70,000*g* for 2 h in an ultracentrifuge (Beckman). The supernatant was concentrated approx 10-fold by membrane pressure ultrafiltration (10,000 mol wt cutoff) (Amicon®; Millipore, Bedford, MA).

Purification of SEAP with Affinity Matrix in Batch Mode

The concentrated SEAP sample was mixed gently with the matrix for 1 h. After SEAP binding with the matrix, the mixture was filtered in a sintered glass funnel to remove the spent supernatant, and the SEAP containing matrix was then rinsed with 5 matrix volumes of column buffer (20 m*M* Tris and 1 m*M* $MgCl_2$, pH 8.0) to wash out the nonbinding protein. The bound SEAP was released by washing with 1 matrix volume of releasing buffer (column buffer that contains 10 m*M* Na_2HPO_4).

Purification of SEAP with Affinity Column

The concentrated SEAP sample (3–5 mL) was loaded into the column (50 × 2.5 cm id) packed with affinity matrix (200 mL). SEAP sample was recycled through the column for 24 h unless otherwise indicated. The column was washed with 1 column volume of column buffer to remove the nonbound protein and then washed with releasing buffer (1–1.5 column volumes) to elute SEAP from the column. SEAP activity in each fraction was assayed to locate the fractions containing pure SEAP. Fractions containing pure SEAP were pooled and concentrated by membrane pressure ultrafiltration (10,000 mol wt cutoff). The protein concentration and sample purity were then determined. All the affinity purifications were conducted at 4°C in a cold room.

Regeneration of Affinity Matrix

After each purification, the affinity matrix was regenerated by washing with 2 L each of 0.2 *M* sodium citrate, 0.5 *M* NaCl (pH 2.5), 0.2 *M* Tris–0.5 *M* NaCl (pH 10.5), and deionized water. Finally, the column was washed with 1 column volume of buffer prior to the next round of purification.

Statistical Analyses

Cell density and viability were determined with a Coulter Counter (Model ZM) interfaced with a Coulter Channellyzer (Coulter Electronics, Hialeah, FL) and by the trypan blue exclusion method, respectively.

SEAP activity was assayed by following the release of *p*-nitrophenol from *p*-nitrophenyl phosphate *(12,15)*. Sample solutions were prepared by mixing equal volumes of supernatant (diluted if needed) and 2X SEAP buffer (2 *M* diethanolamine, 1 m*M* $MgCl_2$, and 20 m*M* L-homoarginine). Two hundred microliters of each resulting sample was placed into a well of a 96-well plate. Substrate solution (20 µL, 120 m*M* *p*-nitrophenyl phosphate in 1X SEAP buffer) was added to each sample well, and the absorbance at 410 nm was measured at 1-min intervals for a period of 15 min using a Dynatech plate reader (Dynatech, Chantilly, VA). Optical density/min values were calculated from linear portions of the resulting curves. These results were then converted to SEAP units per milliliter by using a calibration curve prepared using an alkaline phosphatase standard (Boehringer Mannheim, Indianapolis, IN).

The protein concentration was determined using a Coomassie Plus protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard protein. The purity and molecular weight of SEAP protein were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel) using a Mini-Protean II electrophoresis system from Bio-Rad (Hercules, CA) and silver staining *(16)*.

Results and Discussion

Recombinant SEAP Expression

When the total cell density reached 1 to 2 × 10^6 cells/mL, Sfβ4GalT insect cells were infected with AcMNPV-SEAP at an MOI of 5. The total cell density, cell viability, and SEAP activity are shown in Fig. 1 as a function of time postinfection. Cell density decreased slightly, but cell viability decreased from 99% at 0 h postinfection to approx 75% when the cultures were harvested at 72 h postinfection. SEAP activities reached 1.2 U/mL at the end of cultivation. SDS-PAGE (Fig. 2) of the sample at 72 h postinfection showed that the SEAP had a molecular weight of approx 64 kDa and contained some impurities. Based on the image analysis on SDS-PAGE with UN-Scan-It™ gel software, the purity of SEAP in the cell-free supernatant was estimated to be approx 20%.

Purification of SEAP in a 200-mL Affinity Column

SEAP produced by the Sfβ4GalT cell line was purified in the column containing 200 mL of affinity matrix. Different operation modes and loading amounts were examined to obtain high recovery and purity. Three sets of conditions were tested. First, 5 mL of concentrated SEAP sample was loaded into the column, the sample was recycled in the column at a flow

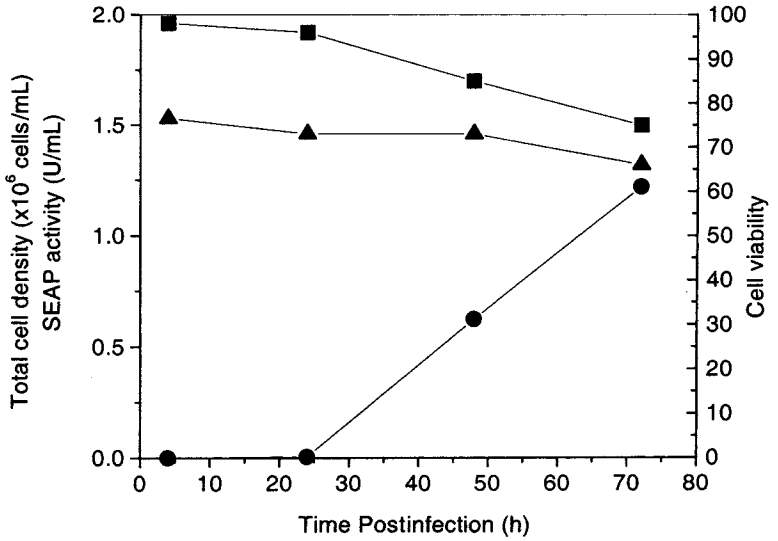

Fig. 1. Expression of recombinant SEAP in the Sfβ4GalT cell line infected with AcMNPV-SEAP at an MOI of 5: total cell density (▲), cell viability (■), and SEAP activity (●) as a function time of postinfection.

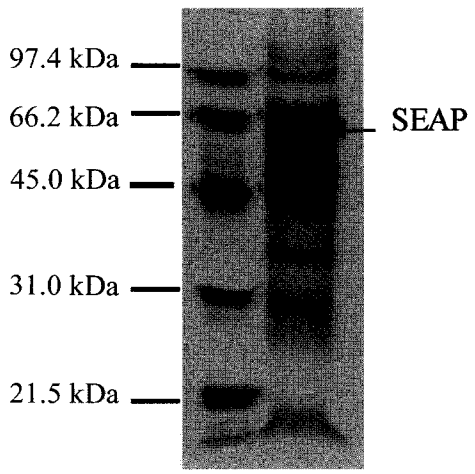

Fig. 2. SDS-PAGE analysis of crude SEAP from the Sfβ4GalT cell line infected with AcMNPV-SEAP. Molecular weight markers are indicated.

rate of 0.25 mL/min for 24 h, the column was washed with 300 mL of column buffer, and SEAP was released by washing the column with releasing buffer at a flow rate of 0.25 mL/min. Second, 3 mL of concentrated SEAP sample was loaded into the column without recycle, the column was then immediately washed with column buffer, and SEAP was eluted by washing the column with releasing buffer at a flow rate of 0.25 mL/min. Third, 2.5 mL of concentrated SEAP sample was loaded into the column

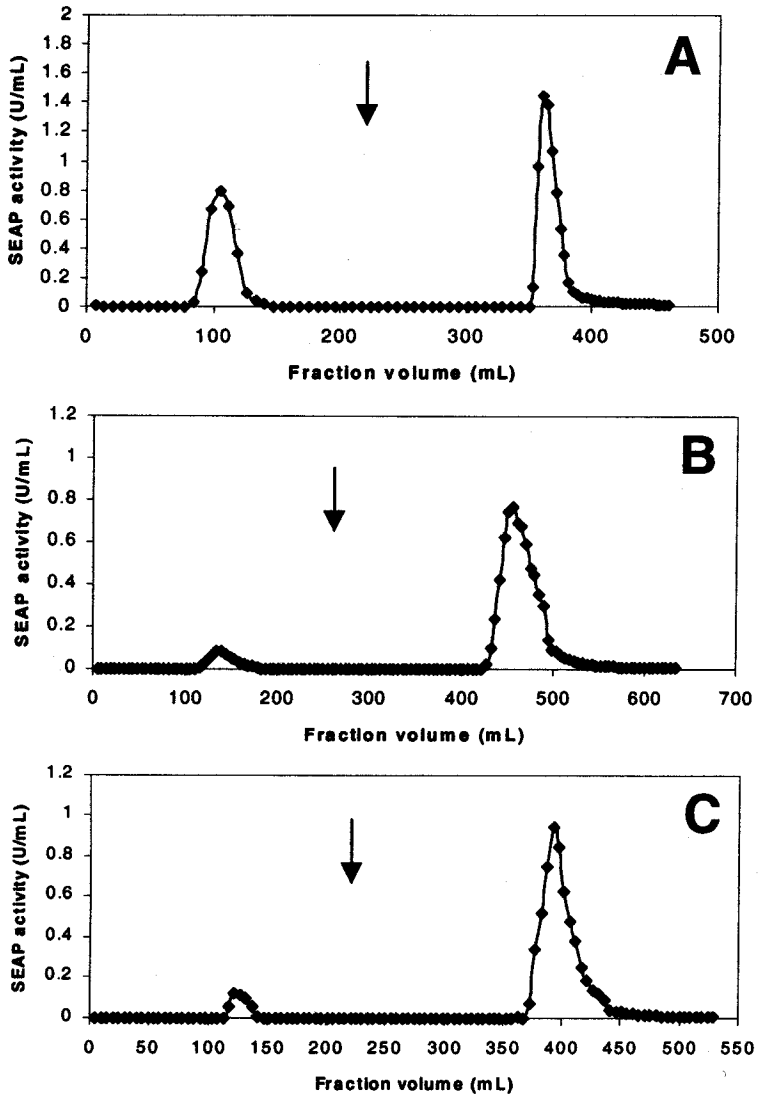

Fig. 3. Chromatograms of SEAP purification in a 200-mL affinity column with different operation conditions. **(A)** Concentrated Sfβ4GalT SEAP sample (5 mL) was loaded on the column and recycled through the column for 24 h; **(B)** concentrated Sfβ4GalT SEAP sample (3 mL) was loaded on the column without recycle; **(C)** concentrated Sfβ4GalT SEAP sample (5 mL) was loaded on the column without recycle. Arrows indicate the point at which column buffer was replaced with phosphate containing releasing buffer.

without recycle, the column was immediately washed with column buffer, and SEAP was eluted by washing the column with releasing buffer at a flow rate of 0.25 mL/min. The chromatograms of the affinity purification are shown in Fig. 3A–C. Based on the SEAP activity released, it was discovered that the activity recovery increased from 57 to 95% as the amount loaded

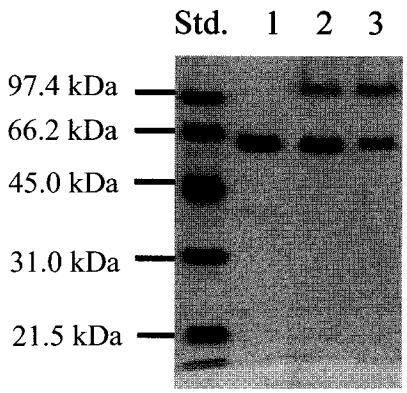

Fig. 4. SDS-PAGE analysis of SEAP purified on the affinity column under different operating conditions. Lane 1, 5 mL of Sfβ4GalT SEAP sample loaded with recycle of the sample through the column for 24 h; lane 2, 3 mL of Sfβ4GalT SEAP sample loaded on the column without recycle; lane 3, 2.5 mL of Sfβ4GalT SEAP sample loaded on the column without recycle. Molecular weight standards are shown in the lanes to the left of the samples.

decreased from 5.0 to 2.5 mL. The purity of the released SEAP was then assessed by SDS-PAGE (Fig. 4). The results showed that recycling the sample through the column affected SEAP purity. SDS-PAGE showing only one SEAP band was observed when the sample was recycled (Fig. 4, lane 1). Without recycle, an additional band at ~98 kDa was also observed when the interacting fraction from the affinity chromatography (Fig. 3B,C) were analyzed on SDS-PAGE (Fig. 4, lanes 2 and 3). This contaminating protein has no phosphatase activity and apparently corresponds to a baculovirus (AcPNPV) protein of unknown function *(17)*. We postulate that this protein, unrelated to SEAP, apparently interacts nonspecifically with the affinity matrix when SEAP concentration is low in the column (Fig. 3B,C), but this interaction can be eliminated by increasing the SEAP concentration and recycling operation (Fig. 3A).

Kinetics Study on SEAP Binding with Affinity Matrix

To understand the effect of SEAP concentration on affinity purification, different amounts of concentrated Sfβ4GalT SEAP (0.1, 0.15, 0.2, 0.3, 0.5, and 1.0 mL) were individually added into 15-mL centrifugation tubes containing 10 mL of affinity matrix. The total reaction volume was made up to 13 mL by adding column buffer. The resulting SEAP activities in liquid phase were 0.46, 0.69, 0.92, 1.38, 2.3, and 4.6 U/mL of liquid, respectively. After gentle mixing in an inverter shaker for 24 h, the supernatant was removed by centrifugation. The SEAP bound to the affinity matrix was washed with 100 mL of column buffer and finally twice with 5 mL of releasing buffer to recover the bound SEAP. SEAP activity and purity in the released samples were assayed. The results showed that the amount of matrix-bound SEAP increased proportionally to the concentration of SEAP

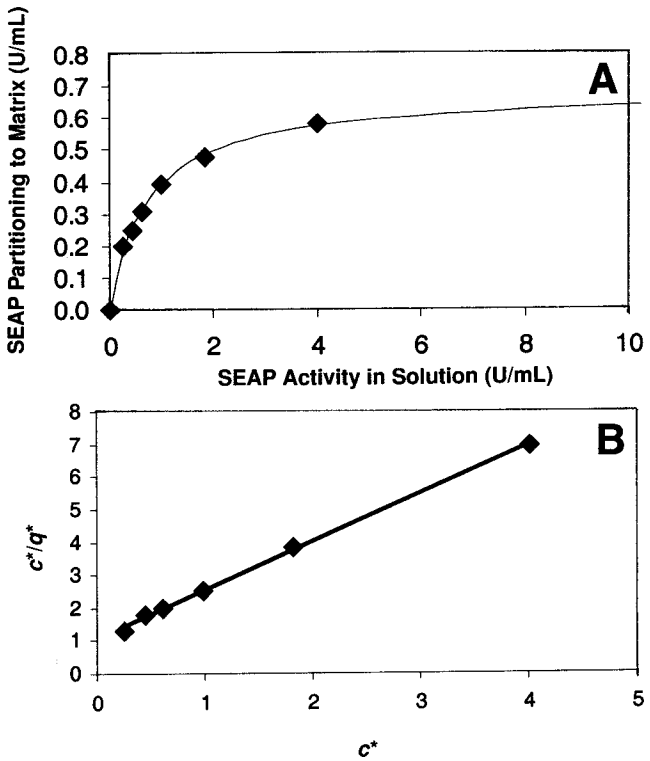

Fig. 5. Kinetics of SEAP affinity binding with the matrix. **(A)** Effects of SEAP concentration on affinity binding (◆). The binding process matches the Langmuir isotherm model well ($y = 0.68x/[x + 0.725]$), shown as a solid line). **(B)** Linearized Langmuir isotherm ($y = 1.47x + 1.07$; $r^2 = 0.998$): c^* is the SEAP concentration in liquid phase (U/mL) and q^* is the SEAP binding amount on the matrix (U/mL of matrix).

in the liquid phase added to the affinity matrix (Fig. 5A). The experimental data obtained in the batch purification fit a Langmuir isotherm quite well ($r^2 = 0.998$) and showed that the maximum carrying capacity is 0.68 U of SEAP/mL of matrix (Fig. 5B). SDS-PAGE demonstrated that the purity of released SEAP improved when increasing amounts of SEAP were loaded onto the affinity matrix (Fig. 6). Some nonspecific binding of protein (Fig. 6, lanes 1–3) on the matrix occurred when low concentrations of SEAP sample were loaded.

These results can be explained by the Langmuir isotherm model, which is representative of a matrix that has a monolayer of sites available for protein binding. Inherent to Langmuir isotherms, operation of a purification in which most binding sites become occupied requires a high concentration of the desired protein in the liquid phase initially, leading to significant product loss. Conversely, if the initial protein concentration in the liquid phase is too low, the likelihood of interaction between impurities and matrix increases, thereby compromising the purity of the protein product. When the Langmuir isotherm is applied to the column purification in

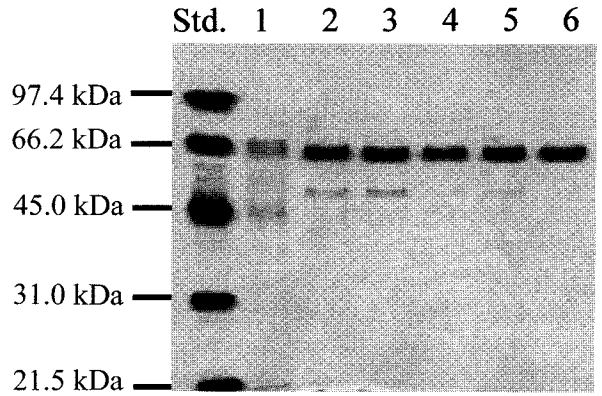

Fig. 6. SDS-PAGE analysis of SEAP purified with 10 mL of affinity matrix on loading with different concentrations of Sfβ4GalT SEAP. Lanes 1–6 correspond to the SEAP activities in liquid phase of 0.46, 0.69, 0.92, 1.38, 2.3, and 4.6 U/mL, respectively. Molecular weight standards are shown in the lanes to the left of the samples.

Fig. 3A and Fig. 3C, we calculate a theoretical recovery of 46 and 91%, respectively, which compares favorably to the actual recovery of 57 and 95%, respectively.

Effects of Binding Reaction Time on Purification

The effect of binding time on purification was examined. Using the experimental procedures previously described, 10 mL of matrix was mixed with 3 mL of column buffer containing 0.3 mL of concentrated Sfβ4GalT SEAP and the binding reaction time was varied (0.5, 1, 2, 6, 12, and 24 h). The SEAP activity and purity of the released samples were then tested (data not shown). The results clearly demonstrated that the binding time had no effect on the recovery (~30%) and the purity (~97%) of the SEAP. These results indicate that the binding process is completed in a very short period, i.e., 30 min or less.

Purification of SEAP with Affinity Matrix in a Flask

The kinetics study demonstrated that the purification procedures including affinity binding, washing, and SEAP release might be completed in a very short time. This suggested that a batch purification of SEAP with affinity matrix might be accomplished rapidly in a flask. A concentrated Sfβ4GalT SEAP sample (6 mL) was mixed gently with 200 mL of matrix for 1 h, the mixture was filtered in a sintered glass funnel to remove the spent supernatant, and the SEAP-matrix was washed with 1 L of buffer to remove the unbound protein. The bound SEAP was then released by washing with 200 mL of releasing buffer. SDS-PAGE showed that the released SEAP was pure and recovery was 41%. The purification steps of Sfβ4GalT SEAP in column and batch purification are summarized in Table 1.

Table 1
Purification of SEAP Produced by Sfβ4GalT Cell Line

Purification mode	Purification steps	Total activity (U)	Total protein concentration (mg)	Specific activity (U/mg)	Fold purification	Recovery (%)
Column purification	Cell-free supernatant	60	9.0	6.7	—	—
	Virus-free supernatant	59	8.3	7.2	1.1	98
	Concentrated SEAP (10,000 mol wt cutoff)	58	8.0	7.3	1.1	97
	Affinity purified in column[a]	33	0.18	183.0	27.0	54
Batch purification	Concentrated SEAP (10,000 mol wt cutoff)	84	8.7	9.7	—	—
	Affinity purified in flask	34	0.23	148.0	15.0	41

[a]Data are based on the purification conditions. Concentrated Sfβ4GalT SEAP sample (5 mL) was loaded on the column and recycled through the column for 24 h. The chromatogram of this affinity purification is shown in Fig. 3A.

Stability of Matrix

The affinity matrix that was prepared has been used for 12 mo in the purification of more than 60 batches of SEAP protein. No decrease in the binding capacity of the affinity matrix has been observed, suggesting that it is very stable under operating conditions.

Acknowledgments

We wish to thank H. Alan Wood for providing the AcMNPV-SEAP and Donald L. Jarvis for providing the Sfβ4GalT cell line. We also gratefully acknowledge support from the National Science Foundation (BES 97-25094).

References

1. Luckow, V. A. (1995), in *Baculovirus Expression Systems and Biopesticides*, Shuler, M. L., Wood, H. A., Granados, R. R., and Hammer, H. A., eds., Wiley-Liss, New York, pp. 51–90.
2. Marz, L., Altmann, F., Staudacher, E., and Kubelka, V. (1995), in *Glycoprotein*, vol. 29a, Montreuil, J., Vliegenthart, J. F. G., and Schachter, H., eds., Elsevier, Amsterdam, pp. 543–563.
3. Jarvis, D. L. (1997), in *The Baculoviruses*, Miller, L. K., ed., Plenum, New York, pp. 389–431.
4. Butters, T. D., Hughes, P. C., and Vischer, P. (1981), *Biochem. Biophys. Acta* **640,** 672–686.
5. Jarvis, D. L, Kawar, Z. S., and Hollister, J. R. (1998), *Curr. Opin. Biotechnol.* **9,** 528–533.
6. Hollister, J. R., Shaper, J. H., and Jarvis, D. L. (1998), *Glycobiology* **8,** 473–480.
7. Millan, J. L. (1986), *J. Biol. Chem.* **261,** 3112–3115.
8. Endo, T., Ohbayashi, H., Hayashi, Y., Ikehara, Y., Kochibe, N., and Kobata, A. (1988), *J. Biochem.* **103,** 182–187.
9. Sofer, G. K. and Nystron, L. E. (1989), *Process Chromatography, A Practical Guide*, Academic, London.
10. Kulakosky, P. C., Shuler, M. L., and Wood, H. A. (1998), *In Vitro Cell. Dev. Biol.-Anim.* **34,** 101–108.
11. Landt, M., Boltz, S. C., and Butler, L. G. (1978), *Biochemistry* **17,** 915–919.
12. Davis, T. R., Trotter, K. M., Granados, R. R., and Wood, H. A. (1992), *Bio/Technology* **10,** 1148–1150.
13. Vijayalakshmi, M. A. (1996), *Mol. Biotechnol.* **6,** 347–357.
14. Matsumoto, I., Mizuno, Y., and Seno, N. (1979), *J. Biochem.* **85,** 1091–1098.
15. Saarinen, M. A., Troutner, K. A., Gladden, S. G., Mitchell-Logean, C. M., and Murhammer, D. W. (1999), *Biotechnol. Bioeng.* **63,** 612–617.
16. Heukesshoven, J. and Denick, R. (1985), *Electrophoresis* **6,** 103–112.
17. Ayres, M. D. Howard, S. C., Kuzio, J., Lopez-Ferber, M., and Possee, R. D. (1994), *Virology* **202,** 586–605.