0273-2289/95/5401-03—0173$07.80

Genetic Engineering to Enhance the Selectivity of Protein Separations

CHARLES E. GLATZ*,[1] AND CLARK F. FORD[2]

Department of Chemical Engineering; and [2]Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011

ABSTRACT

The ability to recover and purify natural and recombinant proteins, and the costs of doing so remain a major task in introducing the potential products of biotechnology. The bases for separation range from specific binding onto tailored reagents to solubility and partitioning behavior governed by a mixed bag of size, charge, and hydrophobicity. In most cases, a combination of methods is used in sequence, and improvements in the selectivity at an early stage can enhance the effectiveness of subsequent (and usually more costly) steps. Genetic engineering provides a means of improving the selectivity within the context of existing separation methods.

By this strategy, improvements in selectivity are sought by bestowing a distinctive property on the protein of interest. The primary sequence of amino acids is altered, such that the protein can be selectively removed from other components of the multicomponent mixture in which such products are commonly found. In this article, the range of these "distinctive properties" and their pairing with various separation methods will be reviewed. Specific examples from our work, in which a distinctive charge is provided via a polypeptide "purification" fusion tail, will be discussed. Separation methods we have used with these fusion proteins are precipitation, two-phase aqueous extraction, reversed micellar extraction, and ion exchange using both resins and membranes.

Index Entries: Protein; separation; purification fusion; precipitation; ion exchange; genetic engineering.

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

INTRODUCTION

Several difficulties are encountered in the recovery of the promising protein products of biotechnology. Chief among these are that the protein is usually present in dilute concentration, that the solution is a complex mixture of cell and media components, that the protein is stable under only a narrow range of conditions, and that final purity requirements may be very high. As a result, the processing volumes are large (relative to the amount of product), many steps (with cumulative yield losses) are required to eliminate other components, several workhorse separation methods are ruled out because of incompatible conditions, and contamination of product with separating agent may not be allowed.

For any product, the hoped for solution to these separation problems is finding an operation that capitalizes on some unique property of the product that will allow it to be plucked directly from the dilute, complex output of the fermenter with great selectivity in a single step. Close approximations would also be welcomed.

For certain proteins, unique properties have been identified that offer the potential for serving as the basis for such a highly selective separation. In most cases, the property is one of binding to a particular ligand. Examples are strepavidin binding to biotin, antibodies binding to antigens, hormones and cytokines binding to receptors, and enzymes binding to cofactors or substrates. For numerous other proteins, a ligand is not readily available. The concept of purification fusions arises from the effort to enable many proteins to be separated by using such affinities.

PURIFICATION FUSIONS

A protein fusion occurs when the genes for expression of two proteins/peptides are linked in-frame with no intervening stop codon. Expression of the gene produces both sequences linked in the same primary sequence. Frequently, each will separately fold to assume whatever native structure it would have possessed as an individual molecule. The strategy for purification fusions is to link the target protein with a peptide able to provide the basis for selective recovery. Hence, the relatively small pool of proteins able to be recovered with high selectivity can be expanded to include any protein that can be fused with one of the easily recovered proteins while maintaining the functionality of each.

A schematic representation of this strategy is shown in Fig. 1 (*1*). Several requisite features of the fusion are shown as well as alternatives in the separation step. Although most of the examples to be cited use binding to a solid packing, Fig. 1 serves as a reminder that there are schemes for using most such binding strategies in other separation techniques, such as precipitation and extraction.

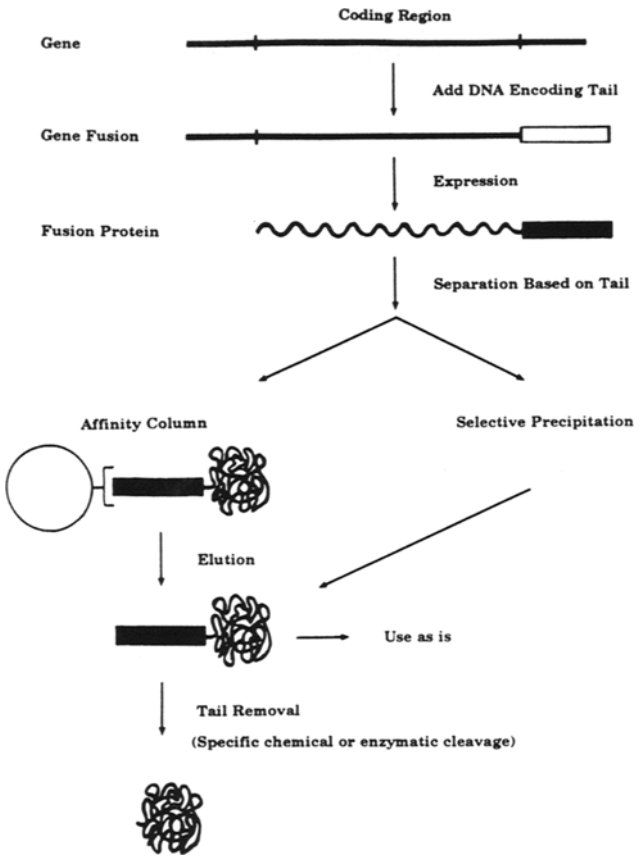

Fig. 1. Construction and use of fusion tails. The tail is shown at the C-terminus in this diagram, but many tails can be engineered at the N-terminus of the target protein. Reprinted with permission of the publisher from Ford et al. (1991), in *Protein Expression and Purification* **2,** 95–107.

The key feature of a purification fusion is that the fused peptide provides a basis for separation. For subsequent use of the recovered protein, it is also necessary that its activity be preserved and, in some cases, that the fusion be cleaved to yield the original target protein. The last feature is particularly important for therapeutic proteins. Table 1 (*1*) gives a summary of many of the fusion "tails" that have been used to recover various target proteins, whereas Table 2 (*2*) gives a sampling of means proposed for cleaving of the tails.

There are two genetic engineering strategies for providing these features. One is to identify restriction sites in the vector carrying the gene for the target protein and prepare oligonucleotides for insertion at the terminus of this gene. The second is to develop a vector with multiple restriction sites between a promoter and the sequence encoding for the cleavage site and purification tail. In the latter case, one would insert the cloned gene

Table 1
Types of Fusion Tails

General type[a]	Size	C- or N-terminus	Separation method[b]
Enzymes			
β-Gal	116 kDa	N, C	IS, IA
GST	26 kDa	N	IS
CAT	24 kDa	N	IS
TrpE	27 kDa	N	HIC
Polypeptide-binding proteins			
SPA	14–31 kDa	N	I-IgG
SPG	28 kDa	C	I-albumin
Carbohydrate-binding domains			
MBP	40 kDa	N	IS (amylose)
SBD	119 aa	C	IS (starch)
CBD	111 aa	N	IS (cellulose)
CBD	128 aa	C	IS
Biotin-binding domain			
Biotination peptide	8 kDa	N	I-avidin
Antigenic epitopes			
recA	144 aa	C	IA
Flag	8 aa	N	IA
Charged amino acids			
Poly(Arg)	5–15 aa	C	IEC, pptn, APS
Poly(Asp)	5–16 aa	C,N	IEC, pptn, APS, RM
Glutamate	1 aa	N	IEC
Other poly(amino acid)s			
Poly(His)	1–9 aa	N,C	IMAC, pptn
Poly(Phe)	11 aa	N	Phenyl-Superose
Poly(Cys)	4 aa	N	Thiopropyl-Sepharose

Reprinted with permission of the publisher from Ford et al. (1991), *Protein Expression and Purification* **2**, 95–107.

[a] βgal, β-galactosidase; GST, glutathione-*S*-transferase; CAT, chloramphenicol acetyltransferase; SPA and SPG, staphylococcal proteins A and G; MBP, maltose-binding protein; SBD and CBD, starch- and cellulose-binding domains.

[b] IA, immobilized antibody; IS, immobilized substrate; HIC, hydrophobic interaction chromatography; I-IgG, immobilized immunoglobulin G; I-albumin, immobilized albumin; I-avidin, immobilized avidin; IEC, ion-exchange chromatography; pptn, precipitation; APS, two-phase aqueous extraction; RM, reversed micellar extraction; IMAC, immobilized metal affinity chromatography.

for the target protein into the purification vector. Ideally, such a vector would have (in the order for a fusion to the C-terminus of the target protein) an inducible, high-expression promoter, a signal sequence for transport to the periplasm or extracellular space, muliple restriction sites in several reading frames, the cleavable linker region, and the purification tail region. Several such vectors are now commercially available.

Table 2
Selective Cleavage Methods

Method	Specificity[a]
CNBr	N-Met*C
Acid	N-Asp*Pro-C
Trypsin	N-Lys/Arg*C
V8 Protease	N-Glu/Asp*C
Collagenase	N-Pro-X*Gly-Pro-C
Clostropain	N-Lys-Arg*C
Enterokinase	N-Asp-Asp-Lys*C
Factor Xa	N-Ile-Glu-Gly-Arg*C
Aminopeptidase I	Glu-Ala-Gly*C (exo)
Carboxypeptidase B	$N^*(Lys)_n/(Arg)_n$ (exo)

Reprinted from ref. 2 by courtesy of Marcel Dekker, Inc.
[a]The side of the amino acid on which cleavage occurs is shown by *. The / indicates that either amino acid may serve as a site. An "X" indicates that many amino acids can occupy that position.

Types of Fusion Tails

Enzymes

Use of an enzyme provides a tail capable of binding to substrate, substrate-analog, cofactor, or anitbody to the enzyme. It also provides a means of assaying for the fusion, i.e., an assay for the enzyme activity. Such fusions are generally larger, sometimes considerably larger, than the target protein, imposing a high metabolic demand on the cell. The usual separation method applied has been affinity adsorption, where the adsorbent is the immobilized ligand. The *TrpE* gene product fusion is an exception that capitalizes on the hydrophobic character of the tail to separate this fusion protein by hydrophobic interaction chromatography.

Polypeptide-Binding Proteins

Staphylococcal protein A binds to the constant region Fc of the mammalian immuloglobulins G. Selected portions of this protein containing the IgG-binding regions have been fused, providing for a smaller tail. Staphylococcal protein G binds to albumin. The two have been used in tandem to provide fusions at each terminus of the target protein (3). This not only provides for sequential purification steps, but also increases the solubility and stability of the target protein.

Carbohydrate-Binding Domains

In this category are the domains of enzymes responsible for substrate binding, but not those domains responsible for catalytic activity. In this way, the size of the fusion is smaller than it would be with the complete enzyme, and the substrate serving as the adsorbent is not hydrolyzed.

Binding to maltose (or crosslinked amylose), insoluble starch, and cellulose has been used. The latter two offer the potential for low-cost adsorbents.

Biotin-Binding Domain

For this strategy, the cell must carry out an in vivo biotinylation of the fusion as well as expressing the fusion. The 8-kDa fusion is a peptide from the 1.3 S subunit of *Propionibacterium shermanii*, which is naturally biotinylated in *E. coli* or yeast. Since binding to tetrameric avidin is generally too strong for elution without denaturation or cleavage, monomeric avidin columns have been developed for easier release.

Antigenic Epitopes

Immunogenic recognition is the basis for separation in this category, but again the emphasis is on finding the smallest peptide able to be recognized. The Flag fusion (4) truly minimizes size in providing both an enzymatic (enterokinase) cleavage site and the antigenic epitope using only eight amino acids. The hydrophilic amino acids of the tail were designed to elicit a strong antigenic response, and a calcium-dependent antibody was selected to provide for elution by removal of calcium.

Charged Amino Acids

Charged fusions are distinguished by their small size and the ready availability of a variety of separation methods based on charge. Ion exchange was the first separation method to be used with charged fusions (5), and other examples of their use will be given below.

Other Poly(amino acid)s

Of these, the metal-binding histidine residues have received the most attention, and tails with varying numbers of histidines, alone or in combination with other amino acids, have been used to recover proteins in native and denatured form. Again adsorption has been the separation method generally used, but proteins with surface histidines added via point mutations have also been recovered by precipitation and extraction (6).

Other Benefits of Purification Fusions

Although their role in simplifying purification was stressed above, the purification fusions have served in two other useful capacities (that do aid recovery as well). These are enhancement of stability and assayability.

Presence of the SPG fusion at the C-terminus stabilized the N-terminal fusion of SPA and human insulin-like growth factor II against proteolytic degradation (3), and the SPA fusion alone has also provided protection (7). Fusions to β-galactosidase have been effective in preserving heterologous proteins from proteolysis (8–11) both as soluble proteins and as inclusion bodies. On the other hand, polyarginine fusions have proven to be prone to degradation by *E. coli* proteases (12,13).

Assaying for the enzymatic activity of an enzyme used as a fusion tail can serve as an indirect assay for the target protein. A variation on this

use is seen in the radioiodination of tyrosine residues in the glutathione-*S*-transferase tail used for the purification of whey acidic protein (*14*). The labeling is also convenient for radioimmunoassay.

Removal of Tails

The cleavage sites in Table 2 fall into several classes. Two provide for chemical cleavage using CNBr or acid. The remainder are enzymatic methods and include endopeptidases with single (trypsin, V8 protease) and multiple (collagenase, enterokinase, Factor Xa) amino acid recognition sites, as well as N- (aminopeptidase I) and C-terminal (carboxypeptidase B) exopeptidases. Most of these cleave at the C-terminal end of the cleavage sequence, which means that only N-terminal fusions will be cleaved to release the authentic target protein.

A cleavage step also means provision must be made for separation of the target protein from the tail and the cleavage enzyme. Where removal of the tail is not necessary, it is possible to turn the retained tail to further advantage. Charged fusions have been used for simultaneous recovery and enzyme immobilization on ion-exchange membranes (*15*).

USE OF CHARGED FUSION TO ENHANCE THE SELECTIVITY OF CHARGED-BASED SEPARATION METHODS

The remainder of this article will illustrate the potential and difficulties associated with one type of purification fusions—charged amino acid fusions—since we have explored their use in our own laboratory. The examples will focus on the application of precipitation by oppositely charged polyelectrolytes and adsorption onto ion-exchange membranes.

Fusion-Tail Nomenclature

The nomenclature we have used to designate the protein and the nature of the charged tail fused to it is as follows. A two-letter abbreviation denotes the enzyme, a C or N denotes the terminus of the enzyme to which the tail is fused, the single-letter abbreviation of the amino acid denotes the charged component of the tail, and a number denotes how many of these charged residues have been added in the tail. Hence, a fusion of five (5) aspartates (D) at the C-terminal end of β-galactosidase (BG) is referred to as BGCD5.

Polyelectrolyte Precipitation

Precipitation is a common method for product concentration and fractionation during the early stages of downstream processing. A variety of precipitating agents have been used, most with high selectively in isolated

cases and a few with promise of being selective in a wider range of applications (*16*). One of the latter approaches is the use of polyelectrolytes of charge opposite to that of the protein being precipitated. Where the target protein has a distinctive charge, good separations have been achieved (*17,18*). Furthermore, the amount of precipitant required is proportional to the amount of protein rather than the volume of protein solution, a distinct advantage for dilute extracts. The use of charged fusions enables a protein of nondistinctive charge to take on distinctive charge and, hence, become a candidate for recovery by polyelectrolyte precipitation.

We have demonstrated this potential by constructing a series of fusions between the carboxyl terminus of β-galactosidase (BGC) and both negatively charged tails (D = aspartate) of the general form Gly-Asp-Pro-Met-Ala-$(Asp)_n$-1-Tyr, where n = 1, 5, 11, or 16 and positively charged tails (R = arginine) of the form Gly-Asp-Pro-Met-Ala-$(Arg)_n$-Tyr, where n = 0, 5, 10, or 15. Except for BGCD16, specific enzymatic activity was within 11% of the wild-type (*13*). The three-dimensional structure of β-galactosidase was recently reported (*19*) and verifies our presumption that the C-terminus is at the surface. β-galactosidase represents a two-fold challenge to this strategy. First, it has an isoelectric point similar to most *E. coli* proteins and hence does not carry a distinctive charge in a cell extract. Second, it is a very large protein (a tetramer with monomeric mol wt of 116,000), so that the tails are only a minor part of the fusion. Precipitations have been done at pH 5.7, where enzyme and poly(asp) tail are both negative, using polyethyleneimine as the precipitant, and at pH 5.4, where the enzyme is still somewhat negative and the poly(arg) tail is positive, using poly(acrylic acid) as the precipitant.

Purified Proteins

Figure 2 shows the enhancement in ability of polyethyleneimine (PEI) to precipitate purified preparations of the charged fusion proteins (*20*). Precipitation of the BGCD11 fusion is completed at low dosages of polyelectrolyte even at fairly high ionic strength (Fig. 3) (*13*). PEI/BGCDx precipitates were redissolved by further increases in ionic strength, and the soluble protein retained enzymatic activity.

The enhanced recovery was also demonstrated in fusions to the monomeric enzyme, glucoamylase (GA) (*21*). This *Aspergillus* enzyme was cloned into yeast and produced as a secreted product. Both N- and C-terminal poly(asp) fusions were produced (GAND5, 7, and 9 and GACD0, 5, and 10, respectively). For both sets of purified fusions, precipitation with PEI occurred to a greater extent at a lower dosage for the tailed enzymes with the GACD10 fusion being most effective (Fig. 4). Enhancements were not as great as for the β-galactosidase cases. Enzymatic activity was retained in the redissolved precipitate.

Cell Extracts

The same series of fusions have been precipitated from *E. coli* extracts and the cell-free broths of the yeast cultures. Figures 5 (*13*) and 6 (*22*) show

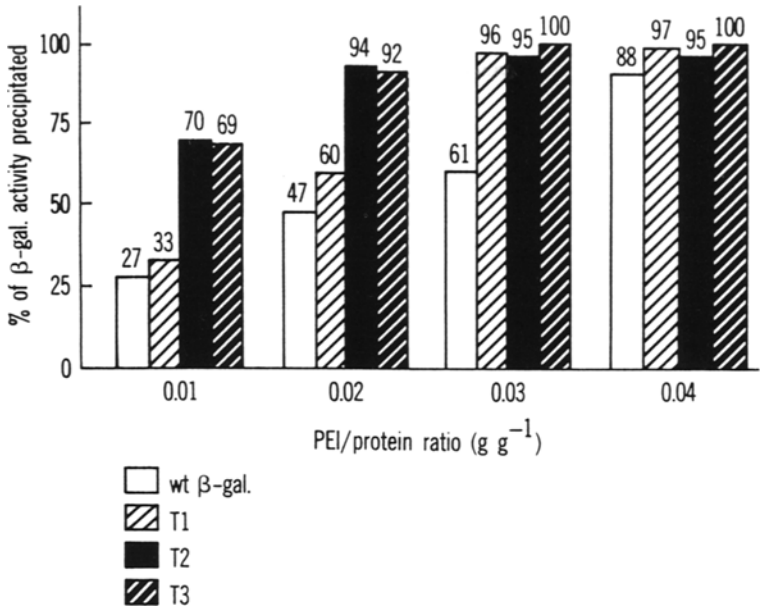

Fig. 2. Precipitation of the fusion proteins BGCD5 (T1), BGCD11 (T2), and BGCD16 (T3) and the wild-type (wt) β-galactosidase (BG) with polyethyleneimine (PEI). Data from duplicate assays deviated < 2% of each other and were averaged. Reprinted with permission of the publisher from Zhao et al. (1990), *J. Biotechnol.* **14,** 273–284.

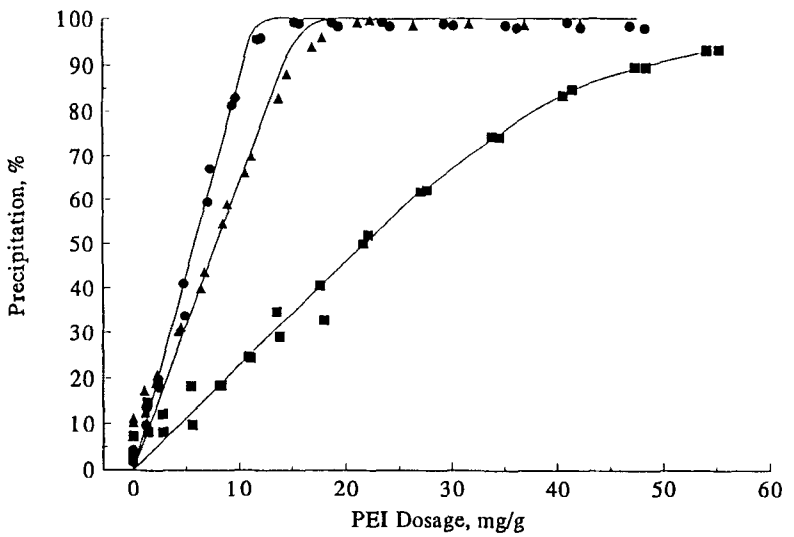

Fig. 3. Comparison of PEI precipitation curves of purified C-terminal (C), β-galactosidase (BG), aspartate (D) fusions in 200 m*M* sodium acetate, pH 5.7: ■, BGCD1; ▲, BGCD5; ●, BGCD11. Reprinted with permission from Niederauer et al. (1994), *Biotech. Prog.* **10,** 237–245. Copyright 1994 American Chemical Society.

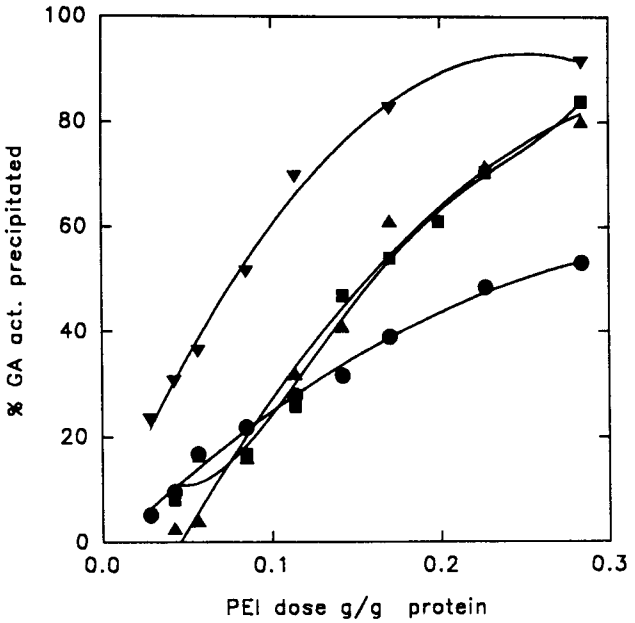

Fig. 4. PEI precipitation of N-terminal (N), glucoamylase (GA), and aspartate (D) fusions expressed by *S. cerevisiae* and purified by starch adsorption. Control version of GA from Pgac9, ●; GAND5, ■; GAND7, ▲; and GAND10, ▼. Reprinted with permission of the publisher from Suominen et al. (1993), *Enzyme Microb. Technol.* **15,** 593–600.

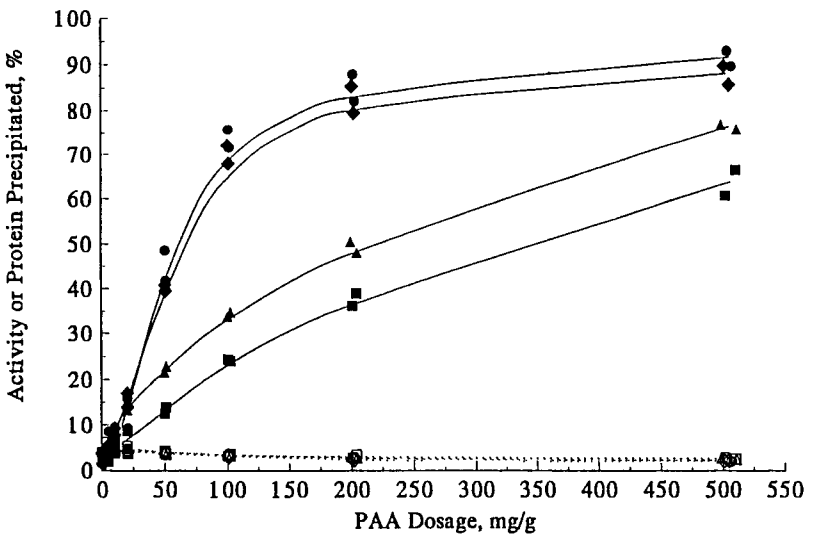

Fig. 5. Poly(acrylic acid) (PAA) precipitation of C-terminal (C), β-galactosidase (BG), arginine (R) fusions from crude extracts of *E. coli* at pH 5.4. Open symbols, total protein; closed symbols, enzymatic activity; ■, □, BGCR0; ▲, △, BGCDR5; ●, ○, BGCR10; ◆, ◇, BGCR15. Reprinted with permission from Niederauer et al. (1994), *Biotech. Prog.* **10,** 237–245. Copyright 1994 American Chemical Society.

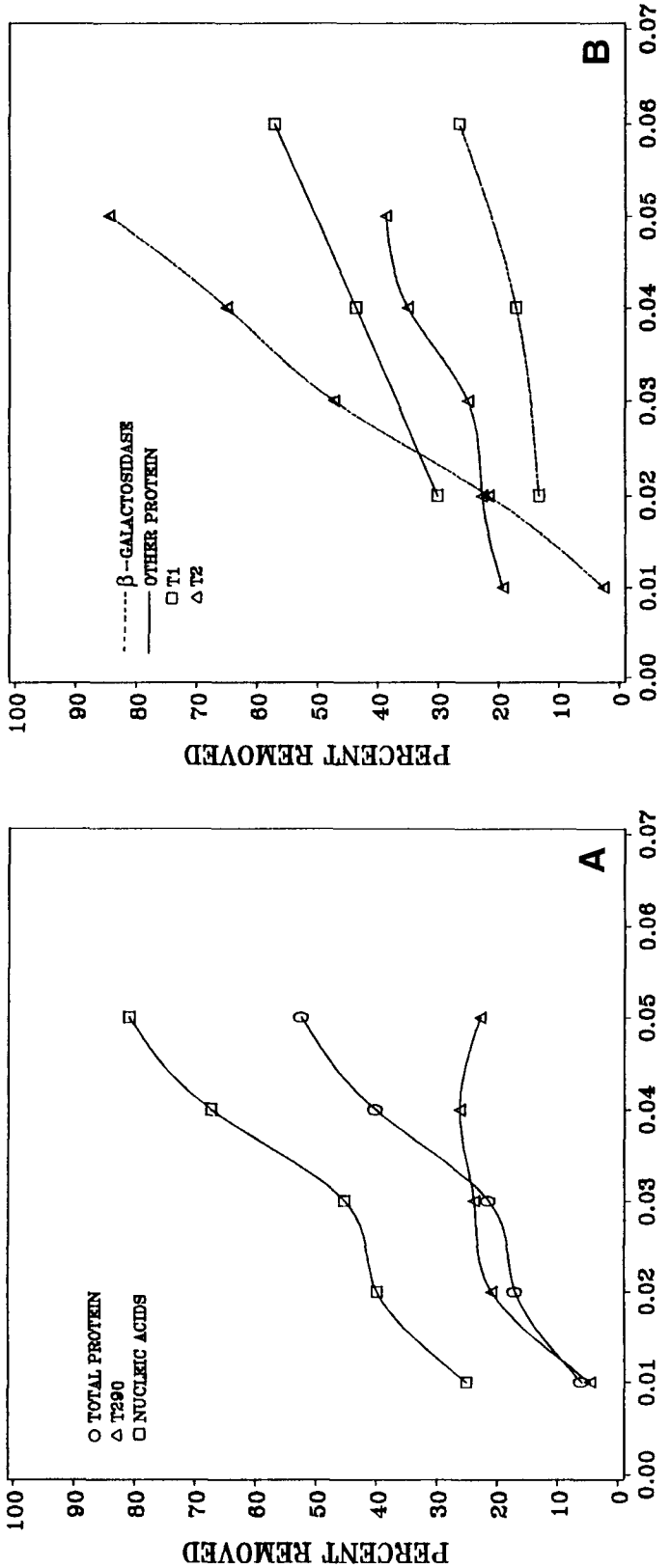

Fig. 6. Comparison of removal of β-galactosidase with the removal of the other proteins in the *E. coli* extract by precipitation with PEI. Reprinted with permission of John Wiley and Sons, Inc. from Parker et al. (1990), *Biotechnol. Bioeng.* **36**, 467–475. (A) PEI precipitation from untreated extract containing the control version of β-galactosidase, T290 (BGCD1) for removal of protein and nucleic acids. Standard deviations are 2.5, 2.0, and 2.5 for percent removal of total protein, β-galactosidase, and nucleic acids, respectively. (B) PEI precipitation from untreated extracts containing the fusion versions of β-galactosidase with polyaspartate tails, i.e., BGCD5 (T1) and BGCD11 (T2). Data for BGCD11 represents the means of two precipitations, whereas those for BGCD5 are a single determination.

that cell components had little effect on the effectiveness of precipitation of the BGCR series of fusions, but that there was significant interference with BGCD series. In the latter case, the key interfering components are the nucleic acids, which are preferentially precipitated by PEI (Fig. 6A). In this case, the tails actually diminish the amount of β-galactosidase being coprecipitated with the nucleic acids (Fig. 6B). For the CR tails, there was the potential for complexation of nucleic acids with the tail, but if this did occur, the addition of poly(acrylic acid) (PAA) was sufficient to disrupt the assocation and precipitate the protein with little nucleic acid contamination. The PAA/BGCR precipitate, however, did not retain activity. The activity loss under these conditions is perhaps not surprising, since β-galactosidase precipitates with loss of activity at pH 5.

The nucleic acid interference with precipitation of the BGCD series was overcome by nuclease digestion of the extract followed by diafiltration of the digest (*22*). After this pretreatment, precipitation followed a very similar course to that with the purified fusions, with the BGCD11 fusion being particularily effective (Fig. 7).

A diafiltration step also proved necessary for recovery of GA fusions from the yeast broth (*21*); some component(s) of the medium appeared to be interfering with the precipitation in this case. However, after diafiltration of the fermentation broth, the fusion tails greatly enhanced recovery, which was quite poor for the untailed enzyme under the same conditions (Fig. 8). GAND10 is not shown in Fig. 8, because the production of the fusion of this length was poor, and reliable precipitation results could not be obtained at the very low concentrations of the enzyme in the broth.

Membrane Ion Exchange

Ion-exchange media could be viewed as immobilized (via crosslinking or adsorption to a solid matrix) polyelectrolytes, and ion exchange could be expected to share some of the selectivity characteristics of polyelectrolyte precipitation. Except for some analytical work with ion-exchange chromotagraphy columns, our ion-exchange studies were performed with ion-exchange membranes. These materials were developed to overcome the mass-transfer limitations of traditional packed columns of porous resins relying on diffusion of the protein into the internal binding sites. The pores of microfiltration-type membranes are derivatized to provide ion-exchange sites lining the pore walls. Feed and eluates are passed through the membrane by pressure-driven convection. We have used both hollow-fiber (HFIEM) (Micro Isonet 1100D, Kinetek System, Inc., St. Louis, MO) and flat-sheet (ActiDisk, FMC Corp., Pine Brook, NJ) membrane configurations (*23*).

Purified Proteins

Both the BGCR and BGCD series of fusions were chromatographed by high-performance ion-exchange chromatography on a column with

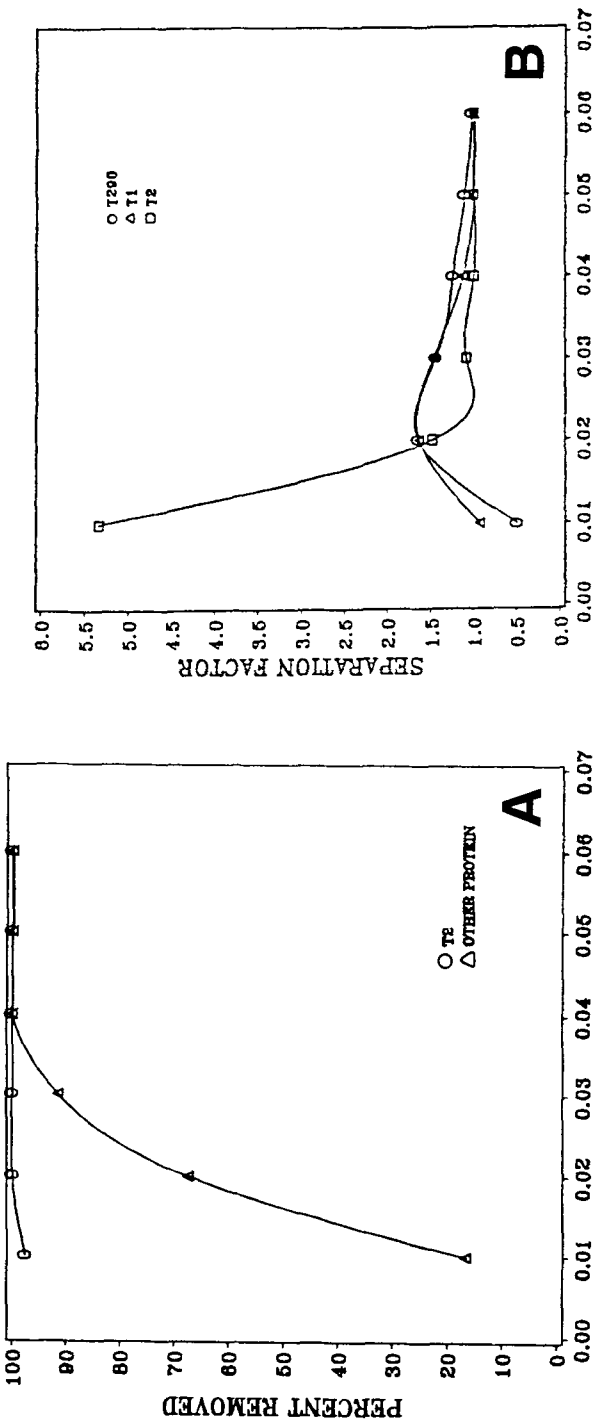

Fig. 7. (A) PEI precipitation on nuclease-treated BGCD11 (T2)-containing *E. coli* extract. Each point represents the mean of two precipitation trials on the same pretreated extract. Standard deviations are 2.2 and 3.7 for percent removal of β-galactosidase and other extract proteins, respectively. (B) Separation factors (ratio of specific activities of the precipitate to that of the extract) for PEI precipitations on nuclease-treated extracts comparing the behavior of BGCD1 (T290, the control), BGCD5 (T1), and BGCD11 (T2). Reprinted with permission of John Wiley and Sons, Inc. from Parker et al. (1990), *Biotechnol. Bioeng.* **36**, 467–475.

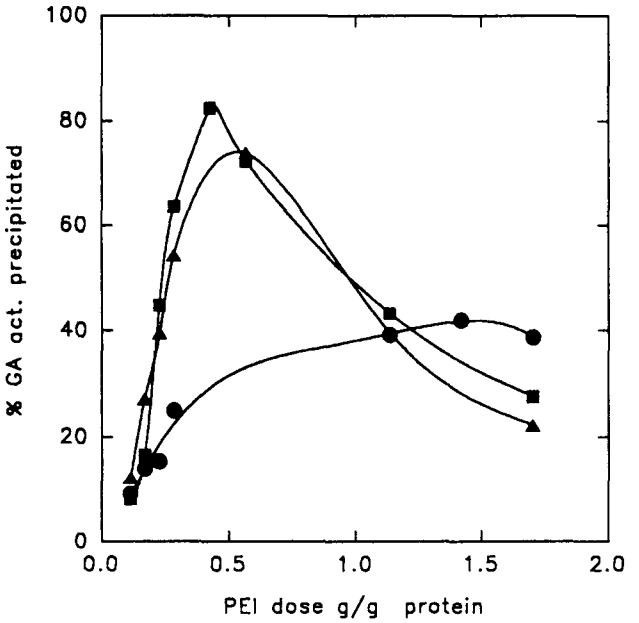

Fig. 8. PEI precipitation of N-terminal (N), glucoamylase (GA), aspartate (D) fusions expressed by *S. cerevisiae* from diafiltered culture supernatants. GAC9 (control), J, GAND5, B, and GAND7, H. Reprinted with permision of the publisher from Suominen et al. (1993), *Enzyme Microb. Technol.* **15,** 593–600.

Toyopearl TSK DEAE 5PW anion-exchange packing. The superimposed elution curves are shown in Fig. 9 (*13*). The positions of the elution peaks in the gradient ranged from 0.1*M* NaCl (in 0.2*M* phosphate buffer, pH 5.7) for BGCD5 to 0.4*M* NaCl for BGCD11. We had earlier observed a somewhat wider range at pH 6.0 using step elutions (0.05*M* increments) from a DEAE Sepharose-6B exchanger (*20*).

Elution from the HFIEM occurred at lower salt concentrations since the Donnan exclusion effects are lower in these large-pore configurations. Step increments in NaCl in 0.089*M* phosphate buffer at pH 7 eluted the wild-type β-galactosidase at 0.05*M* and BGCD11 at 0.2*M* added salt.

Cell Extracts

Before application to the HFIEM, cells were suspended in 50 m*M* Tris-HCl buffer, pH 7.2, containing 10 m*M* $MgCl_2$ and 100 m*M* 2-mercaptoethanol, disrupted by sonication and centrifuged. The supernatant was filtered to remove particulates. The specific activity of each extract was adjusted to the same level by dilution with β-galactosidase-free cell extract (giving an extract in which β-galactosidase comprises 15% of the total protein). Recovery from this cell extract was examined in two modes. The first was simple on-off adsorption, loading at either 0.1 or 0.2*M* ionic strength and eluting at 1*M* ionic strength. The second mode consisted of incremental elution with 0.1*M* steps. All steps were at pH 5.7.

Figure 10 (*23*) summarizes the results for recovery by on-off adsorption. The BGCD11 fusion is the only one capable of being loaded at the

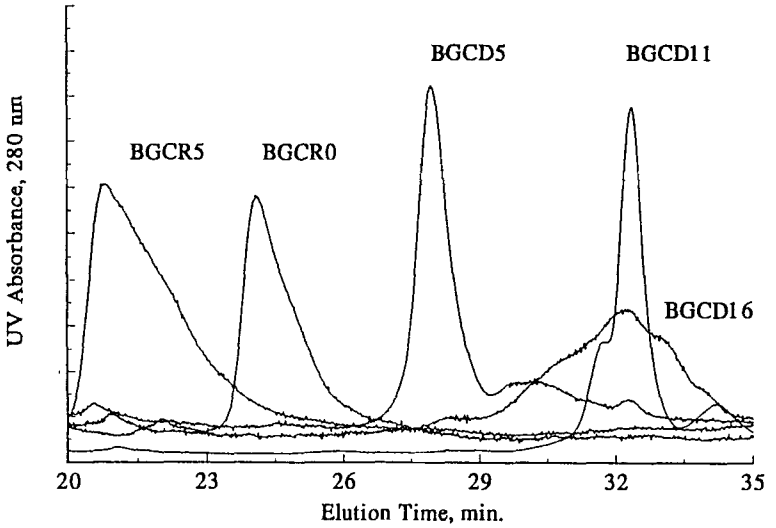

Fig. 9. High-performance ion-exchange chromatography analysis of a series of C-terminal fusions at poly(amino acid) tails to β-galactosidase. A salt gradient was used to elute each protein from a column packed with Toyopearl TSK DEAE 5PW. Here the separate elutions are superimposed. Reprinted with permission from Niederauer et al. (1994), *Biotechnol. Prog.* **10,** 237–245. Copyright 1994 American Chemical Society.

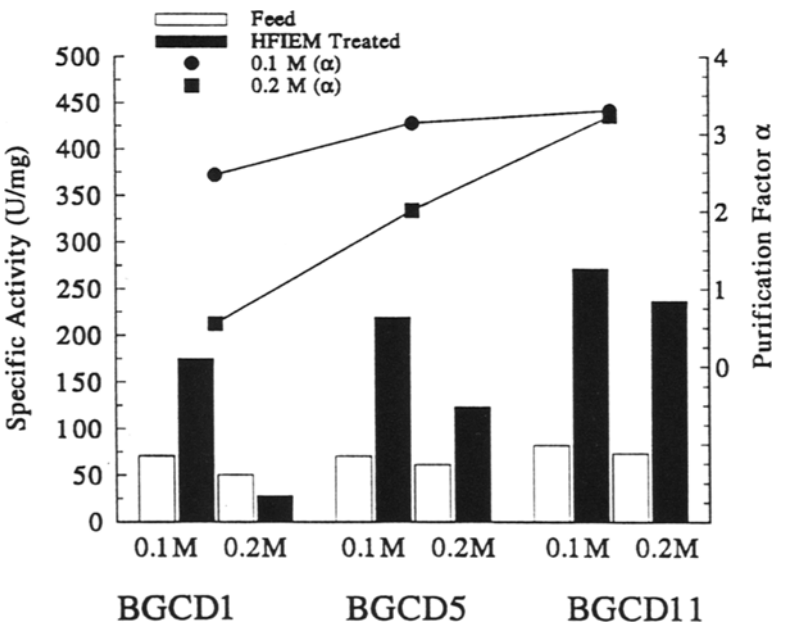

Fig. 10. Binding characteristics of BGCD1, BGCD5, and BGCD11 applied as crude *E. coli* cell extracts (Feed) to hollow-fiber DEAE ion-exchange membranes (Kinetek Micro-Isonet 1100D). Loading conditions were 0.1 or 0.2*M* ionic strength and pH 5.7; bound components were eluted using the same buffer with 1.0*M* NaCl added. Reprinted with permission of John Wiley and Sons, Inc. from Heng et al. (1993), *Biotechnol. Bioeng.* **42,** 333–338.

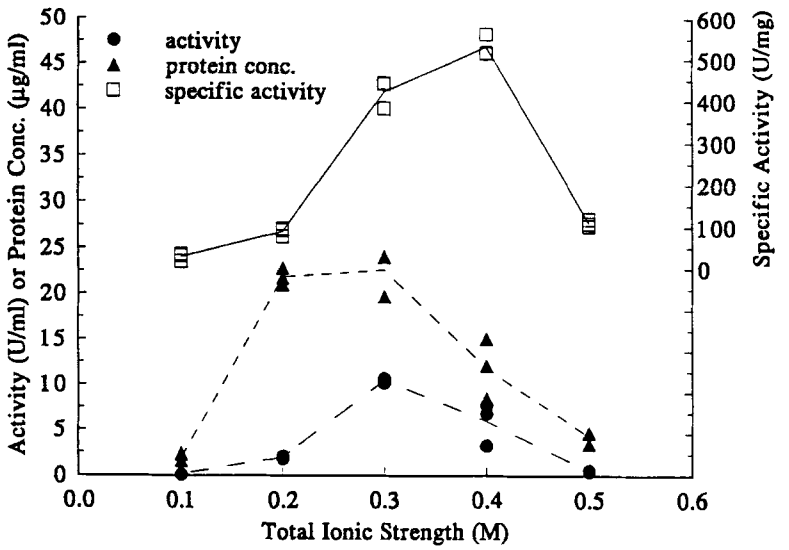

Fig. 11. Activity and protein profiles of bound BGCD11 crude cell extract components eluted from hollow-fiber DEAE ion-exchange membranes (Kinetek Micro-Isonet 1100D) using a step NaCl gradient. Specific activity is calculated as the ratio of β-galactosidase activity (U) to protein content (mg). Loading conditions were 0.1*M* ionic strength and pH 5.7; bound components were eluted using the same buffer with step increments in NaCl concentration. Reprinted with permission of John Wiley and Sons, Inc. from Heng et al. (1993), *Biotechnol. Bioeng.* **42,** 333–338.

higher ionic strength and shows the highest purification factor (ratio of specific activity in the eluate to that in the feed) at the lower ionic strength loading.

In the step elution mode, the tail of the BGCD11 fusion provided the highest binding from the feed and did not diminish the recovery of the bound material. The weak link in the process was the fraction of enzyme in the feed that bound (36% for BGCD11). The binding step was not optimized and could be expected to improve if done at lower ionic strength, if done at lower loadings, or if the pass-through material were recycled. In fact, we were able to obtain much higher recoveries when loading more dilute and lower total quantities of enzymes on the flat sheet membranes to prepare immobilized enzyme reactors (*see below*), an indication of the lower capacities of membrane adsorption systems. The flat-sheet membranes were also used to adsorb GACD5 and GACD10 from fermentation broth that had been concentrated and diafiltered against pH 6.0, 50-m*M* NaAc buffer. In this service, 80% of the glucoamylase activity was bound, and nearly 90% of the bound enzyme was eluted in active form.

The HFIEM elution profiles (23) for BGCD11 are shown in Fig. 11 and compared to those of other β-galactosidase fusions in Fig. 12. The shifts

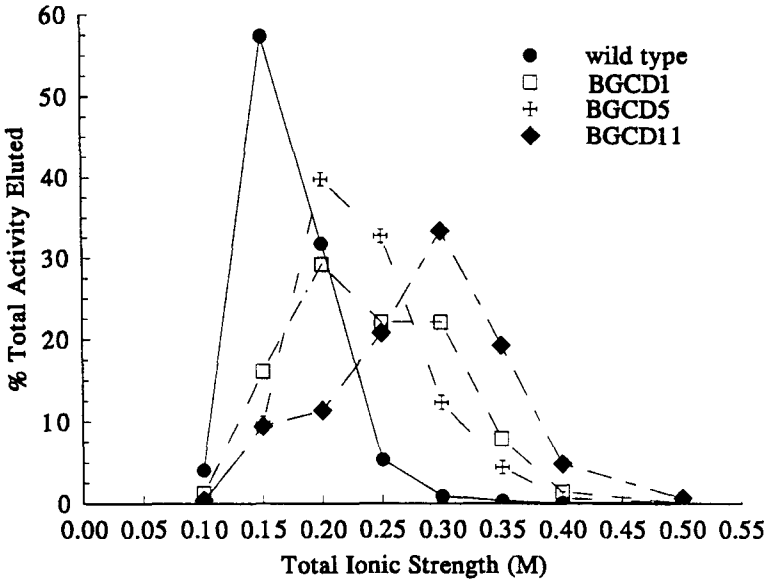

Fig. 12. Step gradient elution profiles of purified β-galactosidase fusions from hollow-fiber DEAE ion-exchange membranes (Kinetek Micro-Isonet 1100D). Loading conditions were 0.1*M* ionic strength and pH 5.7; bound components were eluted using the same buffer with step increments in NaCl concentration. Reprinted with permission of John Wiley and Sons, Inc. from Heng et al. (1993), *Biotechnol. Bioeng.* **42,** 333–338.

in ionic strength required to elute successively longer tails are apparent in the latter figure. The specific activity of the BGCD11 in the 0.4*M* fraction is comparable to that obtained by purification on an affinity column. Similar results were obtained during elution from the flat-sheet membranes (*24*). In the flat-sheet case, the percent of feed β-galactosidase bound was again only about 40% of that in the feed; however, an additional 20% was captured on recycling the wash-through material following an intermediate washing of the column with the 0.1*M* phosphate buffer used to equilibrate the ion-exchange cartridge initially.

Combining Recovery and Immobilization

The flat-sheet membranes were also used to demonstrate possible end use advantages for the fusion tails. The BGCD11 was adsorbed to the membrane directly from the cell extract and then used as an immobilized enzyme reactor (*15*). Compared to the Michaelis-Menten parameters for the wild-type enzyme immobilized in the same fashion, K_m was no different, V_m on lactose was somewhat smaller, and retention was stronger for the tailed enzyme.

SUMMARY

A variety of fusion tails have been used to facilitate protein purification. Most types of such tails have been paired with affinity adsorption for the purification step. In our research, we have worked with a more generic tail type, i.e., one consisting of a sequence of charged amino acids. Fusions possessing these tails are suited for separation via a number of generic charge-based separation methods. We have described the two methods, polyelectrolyte and ion exchange, with which we have worked most extensively and shown where the strategy can be used successfully. We are not limited to just these two separation methods. We have had good results using electrophoretic methods for analytical purposes (*20*). More recently, we have looked at the behavior of such fusions in aqueous two-phase partitioning (*25*) and reversed micellar extraction (*26*). In both of those systems, it appears that the role of charge is not quite as clear-cut as with the methods covered here.

ACKNOWLEDGMENTS

The work of several members of our laboratories has been summarized here, including the cited work of J. Zhao, D. Parker, D., Stachon, M. Niederauer, I. Suominen, and M. Heng. Two undergraduates, Jeff Hunter and Erika Thiem, obtained the data presented for binding and elution of glucoamylase from the flat-sheet ion-exchange cartridge. This article is based on work supported by the National Science Foundation under grant no. BCS-9108583. The government has certain rights in this material.

REFERENCES

1. Ford, C. F., Suominen, I., and Glatz, C. E. (1991), *Protein Expression and Purification* **2,** 95–107.
2. Brewer, S. J., Haymore, B. L., Hopp, T. P., and Sassenfeld, H. M. (1991), in *Purification and Analysis of Recombinant Proteins,* Ramnath, S. and Sharma, S. K., eds., Marcel Dekker, New York, pp. 239–266.
3. Hammarberg, B., Nygren, P.-A., Holmgren, E., Elmblad, A., Tally, M., Hellman, U., Moks, T., and Uhlén, M. (1989), *Proc. Natl. Acad. Sci. USA* **86,** 4367–4371.
4. Hopp, T. P., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L., and Conlon, P. J. (1988), *Bio/Technol.* **6,** 1204–1210.
5. Sassenfeld, H. M. and Brewer, S. J. (1984), *Bio/Technol.* **2,** 76–81.
6. Todd, R. J. (1993), Ph.D. thesis, California Institute of Technology, Pasadena, CA.
7. Moks, T., Abrahmsén, L., Holmgren, E., Bilich, M., Olsson, A., Uhlén, M., Pohl, G., Sterky, G., Hultberg, H., Josephson, S., Holmgren, A., Jörnvall, H., and Nilsson, B. (1987), *Biochemistry* **26,** 5239–5244.

8. Itakura, K., Hirose, T., Crea, R., and Riggs, A. D. (1977), *Science* **198,** 1056–1063.
9. Goeddel, D. V., Kleid, D. G., Bolivar, F., Heyneker, H. L., Yansura, D. G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K., and Riggs, A. D. (1979), *Proc. Natl. Acad. Sci. USA* **76,** 106–110.
10. Shine, J., Fettes, I., Lan, N. C. Y., Roberts, J. L., and Baxter, J. D. (1980), *Nature* **285,** 456–461.
11. Stanley, K. K. and Luzio, J. P. (1984), *EMBO J.* **3,** 1429–1434.
12. Skerra, A., Pfitzinger, I., and Plückthun, A. (1991), *Bio/Technol.* **9,** 273–278.
13. Niederauer, M. Q., Suominen, I., Rougvie, M. A., Ford, C. F., and Glatz, C. E. (1994), *Biotechnol. Prog.* **10,** 237–245.
14. Ullmann, A. (1984), *Gene* **29,** 27–31.
15. Heng, M. H. and Glatz, C. E. (1994), *Biotechnol. Bioeng.* **44,** 745–752.
16. Niederauer, M. Q. and Glatz, C. E. (1992), *Adv. Biochem. Eng./Biotechnol.* **47,** 159–188.
17. Sternberg, M. and Hershberger, D. (1974), *Biochem. Biophys. Acta* **342,** 195–206.
18. Clark, K. M. and Glatz, C. E. (1987), *Biotechnol. Prog.* **4,** 241–247.
19. Jacobson, R. H., Zhang, X.-J., DuBose, R. F., and Matthews, B. W. (1994), *Nature* **369,** 761–766.
20. Zhao, J., Ford, C. F., Glatz, C. E., Rougvie, M. A., and Gendel, S. M. (1990), *J. Biotechnol.* **14,** 273–284.
21. Suominen, I., Ford, C., Stachon, D., Heimo, H., Niederauer, M., Nurmela, H., and Glatz, C. (1993), *Enzyme Microb. Technol.* **15,** 593–600.
22. Parker, D. E., Glatz, C. E., Ford, C. F., Gendel, S. M., Suominen, I., and Rougvie, M. A. (1990), *Biotechnol. Bioeng.* **36,** 467–475.
23. Heng, M. H. and Glatz, C. E. (1993), *Biotechnol. Bioeng.* **42,** 333–338.
24. Thiem, E. and Heng, M. H. (1994), *J. Iowa Acad. Sci.* **101,** 66–69.
25. Luther, J. R. and Glatz, C. E. (1994), *Biotechnol. Bioeng.* **44,** 147–153.
26. Forney, C. E. and Glatz, C. E. (1994), *Biotechnol. Prog.* **10,** 499–503.