Enhancement of polyelectrolyte precipitation through the genetic fusion of charged polypeptides to enzymes

Mark Quinn Niederauer
Iowa State University

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Enhancement of polyelectrolyte precipitation through the genetic fusion of charged polypeptides to enzymes

Niederauer, Mark Quinn, Ph.D.

Iowa State University, 1993
Enhancement of polyelectrolyte precipitation through 
the genetic fusion of charged polypeptides to enzymes

by

Mark Quinn Niederauer

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Chemical Engineering
Major: Chemical Engineering

Approved:

Members of the Committee:

In Charge of Major Work

For the Major Department

For the Graduate College

Iowa State University
Ames, Iowa
1993
# TABLE OF CONTENTS

**GENERAL INTRODUCTION**
- Explanation of Dissertation Format 1
- Literature Review 2

**Salting Out**
- Organic Solvents 6
- Isoelectric Precipitation 7
- Non-Ionic Polymers 8
- Affinity Precipitation 9
- Polyelectrolytes 9
- Other Methods 10
- Enhancement of Separations through Genetic Engineering 10

**PAPER I: SELECTIVE PRECIPITATION**
- LIST OF SYMBOLS AND ABBREVIATIONS 12
- ABSTRACT 14
- INTRODUCTION 16

**CHOICE OF PRECIPITANT**
- Binding and Selectivity 26
  - Affinity interactions 26
  - Polyelectrolytes 26
- Solubility and Precipitation 29
  - Macroligands 30
  - Protein-binding dyes 32
  - Metal ions 32
  - Polyelectrolytes 32

**CONSIDERATIONS OTHER THAN SELECTIVITY**
- Physical Aspects of Precipitation 35
  - Addition of precipitant 35
  - Mixing 37
  - Recovery of the precipitate 38
- Nature of the Extract 38
- Impact of Genetic Engineering 39
- Removal of Precipitant 41

**USE OF SELECTIVE PRECIPITANTS**
- Affinity Macroligands 42
  - Homogeneous macroligands 42
APPENDIX B: ATTEMPTS AT ISOLATING β-GALACTOSIDASE FUSION PEPTIDES 183
APPENDIX C: GACD PRECIPITATIONS 188
APPENDIX D: DERIVATION OF \( \Phi(Z) \) AND \( K_{int} \) 198
GENERAL INTRODUCTION

This work was designed to enhance protein purification using polyelectrolyte precipitation. Genetically altered proteins were used to accomplish this goal. The proteins investigated were β-galactosidase and glucoamylase. β-galactosidase has been altered by the addition of positively and negatively charged peptides (tails) to the carboxyl termini of the protein’s four subunits. Glucoamylase has been modified by the addition of negatively charged peptides to either terminal of the enzyme. The main intentions were to determine the number of added charges which result in the optimal fractionation of the protein from crude lysates, and to model the precipitation behavior based upon data obtained using purified extracts of the recombinant enzymes.

The specific questions which this work was designed to answer are listed below:

1. What is the optimum length of the polypeptide tails for polyelectrolyte precipitation, beyond which no further enhancement is obtained?
2. Does the presence of the tail affect the activity of the enzyme? Parker et al. (1990) found that for additional poly(aspartic acid) residues of 5 and 11, the activity of the recombinant enzyme was equal to that of native β-galactosidase. However, for a tail containing 16 residues, they found that the activity was only about one half that of native β-galactosidase.
3. How much does the presence of the additional localized charges affect the selectivity of the precipitation for charged polypeptide tailed β-galactosidase and glucoamylase? Parker et al. (1990) obtained selectivity factors of up to 5.34 by precipitating the poly(aspartic acid) tailed β-galactosidase with polyethyleneimine. A fermentation which yielded a
lower β-galactosidase content resulted in an even higher selectivity factor of 7.9.

4. How can current models of polyelectrolyte precipitation be modified to account for the high density charge associated with the tails?

**Explanation of Dissertation Format**

The dissertation contains three separate papers which have been published or submitted for publication. The papers are preceded by a literature review which introduces the reader to precipitation. Directly following the papers are the general conclusions. The literature cited in the introduction and in the general conclusions is placed immediately following the general conclusions. The first paper is an in-depth review of the more selective precipitation methods and has already been published (Niederauer and Glatz, 1992). It includes a discussion of the various precipitants, their mechanisms of protein precipitation, further enhancement of precipitation through genetic engineering of the protein, and considerations which must be made in choosing a particular method. The second chapter focuses further on the primary topic of the dissertation: the enhancement of polyelectrolyte precipitation through the genetic engineering of the enzyme β-galactosidase. Experimental work included the characterization and polyelectrolyte precipitation of the various fusion enzymes. The third chapter presents a model to account for the enhancement of precipitation as a result of the charged fusion polypeptides on both multimeric and monomeric proteins.

Four appendices are included which detail work which will either not be published or is published primarily under another author. The first appendix shows results for the estimation of net enzyme charge as a function of the pH for the various fusion proteins investigated. The second appendix discusses efforts made
to cleave the charged fusion polypeptide from the enzyme. The third appendix contains experimental work on the polyelectrolyte precipitation of genetically engineered glucoamylase, a monomeric enzyme. These results were published elsewhere (Suominen et al., 1992). The final appendix contains a more detailed discussion of the derivation for the cooperativity of binding presented in the third paper.

**Literature Review**

The growing demand for less expensive pharmaceuticals and other biochemical products has created a need for the development of more efficient separation processes to lower the cost of purification. Many of these products are enzymes, which often require high yields and purity levels, especially when intended for clinical use. Obtaining a pure product is particularly complicated when one considers the fact that cells consist of a complex mixture of many different components, including cell walls (lipid bilayers), ribosomes, nucleic acids and proteins. To complicate matters further, even in such simple cells as bacteria there are on the order of 1000 different proteins (Georgiou, 1988). It has been noted by several authors that the costs of product recovery often exceed those for the fermentation itself (Bell et al., 1983; Bjurstrom, 1985; Van Brunt, 1985).

The difficulty of product purification is dependent on the fermentation process. If the product is secreted by the microorganism, the product will be fairly dilute in the broth, yet of relatively high purity. On the other hand, if the product is contained within the microorganism, either in the periplasmic space or in the cytosol, the cells must be harvested and subsequently disrupted to recover the product, which will be concentrated, yet relatively impure. In the latter case, the homogenate is often diluted to improve product recovery (Hansen et al., 1971). To
avoid unnecessary product losses, conditions during fermentation and purification must be adjusted so as to avoid denaturation or proteolytic degradation of the product.

Precipitation is one of the primary methods used to achieve fractionation during product recovery. In common practice, precipitation is used during the early stages of downstream processing to achieve partial purification of the product as well as a reduction in volume (Böing, 1982). Other methods used to achieve product purity include chromatography, electrophoresis, and ultrafiltration. All of the above methods are used to varying degrees on analytical, preparative, and industrial scales. To determine the appropriate method for a given goal, the following factors need to be taken into consideration:

1. Concentration of final product
2. Selectivity of the method
3. Product yield
4. Economics of the method
5. Retention of product activity (enzymatic)
6. Amenability to operation at desired scale
7. Safety of the method
8. Amenability to continuous operation
9. Toxicity of processing chemicals if the product is destined for use in pharmaceuticals or consumable products
10. Compatibility with other processing methods.

In view of these factors, precipitation is an excellent choice of method for product purification. Precipitation is currently the most widely used method in the isolation of proteins. If the product is found in the precipitant, both enrichment and
concentration are accomplished in one step. This in turn results in lowered costs due to the subsequent processing of smaller volumes. Most precipitating agents are inexpensive and recoverable, lending to favorable economics. Precipitation processes can be scaled up, are readily suitable to continuous operation, and the activity of the protein may be retained.

Traditional precipitation techniques have the reputation of having relatively low selectivities when compared chromatographic separation techniques (Bonnerjea et al., 1986, Niederauer and Glatz, 1992). Newer techniques such as polyelectrolyte and affinity precipitation achieve product purities comparable to those achieved by chromatographical techniques. The advent of genetic engineering further enables the targeting of specific proteins for separation through the fusion of specific handles to the proteins for binding. This work investigates the use of polyelectrolytes to selectively separate genetically engineered proteins. An overview of the various methods of precipitation is useful in gaining an understanding as to the relative advantages and disadvantages of each.

The various methods of protein precipitation outlined here all occur through two basic mechanisms: a change in either the solvent characteristics or the surface properties of the solute resulting in aggregation. The surface of a typical globular protein consists of charged (positive and negative), polar (hydrophilic), and nonpolar (hydrophobic) regions. The differing proportions of these regions on various proteins determines their solubilities in the surrounding solvent. The protein remains soluble if the thermodynamics favor being surrounded by solvent rather than aggregating with other proteins to form a solid phase.

The different methods available for precipitation can be divided into three groups:
1. Change in the solution characteristics through the addition of an organic solvent, non-ionic polymer, or salt.

2. Neutralization of the effective surface charge of the protein and thereby a decrease in its solubility, through the addition of small amounts of acid, base, or ionic polymer.

3. Affinity precipitation, which uses a ligand to bind to the target protein to effect its precipitation.

The methods outlined here are merely overviews of the particular processes, meant to familiarize the reader with the various means by which precipitation can occur. For a more in depth summary of precipitation processes, the reader is referred to Glatz (1989) and Niederauer and Glatz (1992).

Salting Out

Of precipitation methods, salting out is the most frequently used method of enzyme purification. Empirical relations are available which relate protein solubility to ionic strength. During salting out, the energy balance becomes dominated by hydrophobic interactions. The salt ions displace water molecules in the solution which had previously surrounded hydrophilic areas of the protein and shielded against hydrophobic attraction between the proteins (Scopes, 1987). The unshielded hydrophobic areas are thus attracted to each other, leading to aggregation and finally precipitation. The change in solvent structure responsible for precipitation has been modeled by Melander and Horvath (1977) as a change in surface tension. Their model successfully describes many of the features of solubility as a function of salt concentration.

Ammonium sulfate is used most often for salting out. It is inexpensive, highly soluble, stabilizes proteins, has a low heat of mixing, and acts as a
preservative against bacterial growth. It can be added in stages so as to achieve fractionation of a protein mixture. The disadvantages of using ammonium sulfate include its corrosiveness towards metal and concrete (Zadow and Hill, 1975) and disposal problems. Residuals must be removed from the solution in succeeding processing steps, since even low levels of ammonium sulfate are prohibited in pharmaceutical and food products. As with other methods, the method of contacting is important in protein precipitation (Foster et al., 1976; Bell et al., 1983).

**Organic Solvents**

The addition of an organic solvent reduces the solvation strength of the solution by lowering the dielectric constant. This effect is most pronounced at the isoelectric point. Solvents used include ethanol, methanol, isopropanol, and acetone: they fulfill the criteria of "unlimited solubility in water" and are unreactive with proteins. They can be recycled, yet must be handled carefully due to their flammability. Precipitation is performed primarily at lower temperatures, -10°C to 10°C, to avoid protein denaturation (Sternberg and Hershberger, 1974). Tight control of pH and ionic strength are needed to obtain reproducible fractionation. Additional benefits of using organic solvents are that they have a bactericidal effect at the concentrations (>10%) needed for precipitation, and that they can be easily recovered and recycled due to their high volatility. On the downside, precipitation must often be carried out in closed vessels due to the flammability of the solvents.

**Isoelectric Precipitation**

Isoelectric precipitation is the cheapest and simplest of the precipitation methods. Precipitation is initiated by altering the pH of the solution so that the target protein is electrostatically neutral - the isoelectric point of the protein. The attractive forces dominate, leading to aggregation of the protein and a reduction in
its solubility. In a mixture of proteins the isoelectric point is synonymous with the pH of minimum solubility.

An advantage to isoelectric precipitation is that mineral acids such as phosphoric acid can be used. They are inexpensive, are used in low concentrations, and are allowed for use in pharmaceutical and food product recovery. Care must be taken, however, to carefully control the addition of acids to avoid protein denaturation (Hill and Zadow, 1974). The choice of acid can influence the susceptibility of a protein to denaturation, according to where the ion lies in the Hofmeister series (Rothstein et al., 1977). Other methods of precipitation are most effective when performed at the isoelectric point. This method suffers from poor fractionation potential, which is most likely due to protein-protein interactions.

Non-Ionic Polymers

Two models of precipitation by non-ionic polymers have been found to lead to the same form of the solubility equation (summarized in Juckes, 1971): the thermodynamic theory of Ogston and the volume displacement theory conceived by Laurent. The latter theory is supported by the observation that lower concentrations (5 to 10 wt%) of polyethylene glycol (PEG) are needed to precipitate larger molecules than are needed to precipitate smaller molecules (up to 20 wt%). PEG is the favored choice for non-ionic precipitation due to its reasonable cost, moderate viscosity, and high solubility in water. The polymer also stabilizes proteins and can be used at ambient temperatures.

A disadvantage is that the polymer solutions required (40 to 50 wt%) possess a relatively high viscosity. Furthermore, the costs of most non-ionic polymers are high compared to the aforementioned precipitating agents, and recovery of the polymer is often difficult. Traces of polymers left in the solution may also interfere
with successive processing steps. The fractionation potential is generally poor due to protein-protein interactions (Gault and Lawrie, 1980).

**Affinity Precipitation**

The most specific precipitation method is that of affinity precipitation. An example of affinity precipitation occurs through the use of bifunctional ligands which are biospecific to a region of the target protein (Larsson and Mosbach, 1981). Since the precipitation is biospecific, very high purifications can be achieved. Yet since the ligands are very costly, their recovery is of high importance and they are used primarily in the recovery of high value products such as pharmaceuticals. A more in depth discussion of this type of precipitation can be found in the following chapter.

**Polyelectrolytes**

Polyelectrolytes are water-soluble polymers consisting of charged repeat units, resulting in an expanded random coil formation (Rice and Harris, 1954; Tsvetkov et al., 1964). Examples of polyelectrolytes commonly used include the polyanions poly(acrylic acid) (PAA) and carboxymethyl cellulose (CMC), and the polycation poly(ethyleneimine) (PEI). The net effect of the electrostatic repulsion between protein molecules is minimized upon complexation between the polyelectrolyte and protein (Sternberg, 1970). Advantages of the method include high removal efficiencies and retention of enzymatic activity (Sternberg and Hershberger, 1974; Clark and Glatz, 1990). Very low amounts of polyelectrolyte (0.05-0.10 %wt./vol.) are required and the fractionation potential is good (Scopes, 1987). Although the polymers are expensive, reclamation and recycling can be accomplished (Naeher and Thum, 1974; Bozzano, 1989). A disadvantage is the
associated increase in susceptibility of the protein to thermal denaturation (Hidalgo and Hansen, 1971; Gekko and Noguchi, 1978).

Other Methods

Additional precipitating agents include protein binding dyes and multivalent cations. Precipitation in both cases results from ionic associations. The protein dye complex results in a more hydrophobic surface which is insoluble (Bertrand et al., 1985). An example of a binding dye is Rivanol, an organic cation which has been used with serum proteins (Rothstein et al., 1977). Multivalent metal ions such as Ca++ and Mn++ result in ionic bridges between protein molecules (Glatz, 1989).

Another method of precipitation is possible if the target protein is heat stable. The desired protein can be purified by heating the solution to denature other proteins, which then precipitate and can be removed by centrifugation (Scopes, 1987; Takesawa et al., 1990).

Enhancement of Separations through Genetic Engineering

The majority of studies on the enhancement of separations through the genetic engineering of proteins have concentrated on the use of chromatographic separations. Application of these techniques to precipitation is simple since the methods of binding, and therefore selectivity, are essentially identical. Many different types of fusions have been prepared, including metal ion binding, affinity ligands, and charged polypeptides. These different types of fusions are discussed in more detail in Paper I. As an example of the enhancement of charge based separations, ion exchange chromatography has been shown to enhance the recovery of a genetically modified small protein, β-urogastrone, containing positively charged fusion tails (Sassenfeld and Brewer, 1984; Brewer and Sassenfeld, 1985).
Similar results were obtained for aspartic acid tailed \( \beta \)-galactosidase fusions (Zhao et al., 1990).

Parker et al. (1990) and Zhao et al. (1990) have both experimented with poly(aspartic acid) tailed \( \beta \)-galactosidase in precipitation with poly(ethyleneimine) (PEI). Parker et al. concentrated their precipitation work on cell extracts from disrupted *Escherichia coli*. The precipitations of these cell extracts with PEI exhibited interference from nucleic acids. Earlier work had shown selective removal of nucleic acids from extracts by PEI precipitation, but with significant (15-20\%) coprecipitation of proteins (Atkinson and Jack, 1973). The effect has been supported by charge-balance calculations which have shown the precipitation to result from the binding of negatively charged nucleic acids to positively charged proteins and PEI (Cordes, 1987). Zhao et al. performed precipitations using purified proteins. Both Parker et al. and Zhao et al. showed that the charged polypeptide fusions resulted in enhanced precipitation over that of the wild-type enzyme. As the number of charges on the fusion polypeptides increased, lower polyelectrolyte dosages were required to effect the same precipitation.
PAPER I: SELECTIVE PRECIPITATION
SELECTIVE PRECIPITATION

by

Mark Q. Niederauer and Charles E. Glatz

Department of Chemical Engineering

Iowa State University
Ames, Iowa 50011

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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Empirical constant</td>
</tr>
<tr>
<td>AS</td>
<td>Hydroxypropyl methylcellulose acetate succinate</td>
</tr>
<tr>
<td>B</td>
<td>Empirical constant</td>
</tr>
<tr>
<td>C</td>
<td>Empirical constant</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethyl cellulose</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DMPE-B</td>
<td>Dimyristoylphosphatidylethanolamine-biotin</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid</td>
</tr>
<tr>
<td>I</td>
<td>Ionic strength</td>
</tr>
<tr>
<td>K</td>
<td>Equilibrium dissociation constant for binding</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactose dehydrogenase</td>
</tr>
<tr>
<td>GMA</td>
<td>Glycidyl methacrylate</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunogammaglobulin</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactose dehydrogenase</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NASI</td>
<td>N-acryloxysuccinimide</td>
</tr>
<tr>
<td>NIPAM</td>
<td>N-isopropyl acrylamide</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly(acrylic acid)</td>
</tr>
<tr>
<td>PAB</td>
<td>p-aminobenzamide</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>Poly GAB</td>
<td>PAB covalently attached to the NIPAM-GMA copolymer</td>
</tr>
</tbody>
</table>
Poly SAB  |  PAB covalently attached to the NIPAM-NASI copolymer  
STI      |  Soy trypsin inhibitor  
WBA      |  Tungstoboric acid  
WGA      |  Wheat germ agglutinin  
WPA      |  Tungstophosphoric acid  
Z        |  Net charge  
α        |  Separation factor  
γ        |  Purification factor  
**Subscripts**  
b        |  Binding site  
c        |  Concentration  
p        |  Protein  
m        |  Michaelis-Menton
Precipitation methods which offer specificity towards a target protein can greatly decrease the number of steps required to achieve product purity. Techniques which offer varying degrees of specificity include the use of polyelectrolytes, biospecific affinity ligands, metal ion affinity ligands, protein-binding dyes, salts, non-ionic polymers, and solvents. The success of these techniques can often be enhanced through the use of genetic engineering. This review surveys the current status of specific precipitation techniques.
INTRODUCTION

Precipitation is one of the primary methods used to achieve concentration during product recovery. In common practice, precipitation is the separation technique most often used during the early stages of downstream processing to achieve partial purification of the product as well as a reduction in volume.\textsuperscript{1,2} Since the product is most often found in the precipitant, both enrichment and concentration are accomplished in one step. Concentration reduces costs because smaller volumes go on to further processing. The precipitating agents which have greater selectivity tend to be more expensive, but may be recyclable. Precipitation processes can be scaled up, are readily suitable to continuous operation, and can be done such that enzymatic activity is retained.

Precipitants include acids or bases (isoelectric), salts (salting-out), organic solvents, nonionic polymers, polyelectrolytes, protein-binding dyes, multivalent metal ions, and homogeneous and heterogeneous affinity ligands. Examples of the purifications obtainable using these agents can be seen in Table 1. The focus of this review will be on the selectivity conferred by those techniques towards the protein(s) they are targeting. To quantitatively characterize the selectivity of a precipitation, three different definitions are typically used: the purification factor, the separation factor, and a variant of the separation factor. Any of these definitions are suitable for comparing the selectivities of various precipitants since they can easily be calculated from experimental data. The purification factor ($\gamma$) is defined as

$$
\gamma = \frac{[\text{target protein}]_{\text{in precipitate}}}{[\text{total protein}]_{\text{in precipitate}}} \times \frac{[\text{target protein}]_{\text{in extract}}}{[\text{total protein}]_{\text{in extract}}} \quad (1)
$$
Table 1.1. Precipitation for fractionation of proteins

<table>
<thead>
<tr>
<th>TARGET PROTEIN(S)</th>
<th>ORIGINAL MATERIAL</th>
<th>YIELD</th>
<th>PURIFICATION FACTOR</th>
<th>SEPARATION FACTOR</th>
<th>PRECIPITATING AGENT</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ISOELECTRIC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolyl-tRNA Synthetase</td>
<td>mung bean extract</td>
<td>71</td>
<td>2.6</td>
<td>-</td>
<td>Acid</td>
<td>3</td>
</tr>
<tr>
<td>Two Proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycinin (I)³</td>
<td>soy bean extract</td>
<td>75</td>
<td>1.9</td>
<td>-</td>
<td>Acid</td>
<td>4</td>
</tr>
<tr>
<td>β-Conglycinin (II)</td>
<td></td>
<td>50</td>
<td>1.6</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SALTING-OUT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyceral phosphate dehydrogenase</td>
<td>muscle extract</td>
<td>74</td>
<td>2.6</td>
<td>-</td>
<td>(NH₄)₂SO₄</td>
<td>5</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td><em>S. cerevisiae</em> extract</td>
<td>90</td>
<td>3.8</td>
<td>-</td>
<td>(NH₄)₂SO₄</td>
<td>6</td>
</tr>
<tr>
<td><strong>ORGANIC SOLVENT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Various Proteins</td>
<td>blood plasma</td>
<td>22</td>
<td>2.4</td>
<td>-</td>
<td>EtOH</td>
<td>7</td>
</tr>
<tr>
<td>Fibrinogen (I)</td>
<td></td>
<td>51</td>
<td>1.8</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Globulins (II &amp; III)</td>
<td></td>
<td>33</td>
<td>2.1</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Globulins (III)</td>
<td></td>
<td>87</td>
<td>1.5</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin (S-(II &amp; III))</td>
<td></td>
<td>73</td>
<td>1.7</td>
<td>-</td>
<td>Acetone</td>
<td>8</td>
</tr>
<tr>
<td>Phytase</td>
<td><em>A. carneus</em> filtrate</td>
<td>73</td>
<td>1.7</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NONIONIC POLYMER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol oxidase</td>
<td>mycelial extract fraction</td>
<td>77</td>
<td>1.7</td>
<td>-</td>
<td>PEG</td>
<td>9</td>
</tr>
<tr>
<td>α-glucosidase</td>
<td><em>S. Carlsbergensis</em> extract</td>
<td>70</td>
<td>5.4</td>
<td>-</td>
<td>PEG</td>
<td>10</td>
</tr>
<tr>
<td>α-glucosidase</td>
<td><em>S. Carlsbergensis</em> extract</td>
<td>50</td>
<td>6.9</td>
<td>-</td>
<td>PEG</td>
<td>10</td>
</tr>
<tr>
<td>Various Proteins</td>
<td>pig liver extract</td>
<td>80</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td></td>
<td>90</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
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<tr>
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### Table 1.1. (cont.)

**HOMOGENEOUS MACROLIGANDS**

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<tr>
<th>MACROLIGANDS</th>
<th>ox heart extract</th>
<th>91</th>
<th>40</th>
<th>180</th>
<th>Bis-NAD</th>
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<td>Biocytin-dextran</td>
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<table>
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<tr>
<th>Avidin</th>
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**HETEROGENEOUS MACROLIGANDS**

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<th>MACROLIGANDS</th>
<th>pig heart extract</th>
<th>50</th>
<th>7.0</th>
<th>-</th>
<th>Con A ligand</th>
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<td>Lactase Dehydrogenase</td>
<td>beef pancreas extract</td>
<td>79</td>
<td>5.6</td>
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<td>Affinity</td>
<td>37</td>
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<tr>
<td>Trypsin</td>
<td>mixture @ 50% each</td>
<td>82</td>
<td>1.95</td>
<td>41</td>
<td>Poly GAB</td>
<td>91</td>
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<tr>
<td>Two Proteins</td>
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<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>Chymotrypsin</td>
<td>mixture @ 50% each</td>
<td>74</td>
<td>1.92</td>
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<td>Poly SAB</td>
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<td>-</td>
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<tr>
<td>Chymotrypsin</td>
<td>Trypsin</td>
<td>93</td>
<td>5.5</td>
<td>-</td>
<td>STI-chitosan</td>
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<tr>
<td>Wheat germ agglutinin</td>
<td>porcine pancreas extract</td>
<td>70 11</td>
<td>e</td>
<td></td>
<td>Chitosan</td>
<td>95</td>
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<tr>
<td></td>
<td>wheat germ extract</td>
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<td>e</td>
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<td>-</td>
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<td>Protein A ligand</td>
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<td>DMPE-B</td>
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**PROTEIN-BINDING DYES**

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<th>DYES</th>
<th>rabbit muscle extract</th>
<th>97</th>
<th>6.1</th>
<th>e</th>
<th>Triazine dye</th>
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<tr>
<td>Lactose dehydrogenase</td>
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<td>60</td>
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<td>PEG-triazine</td>
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<td>Glucose-6-phosphate dehydrogenase</td>
<td>3-phosphoglycerate kinase</td>
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Table 1.1. (cont.)

<table>
<thead>
<tr>
<th>METAL ION AFFINITY</th>
<th>POLYELECTROLYTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human hemoglobin and pure enzymes</td>
<td>egg white @ 25%</td>
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<tr>
<td>Whale myoglobin</td>
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<tr>
<td>METAL ION AFFINITY</td>
<td>POLYELECTROLYTES</td>
</tr>
<tr>
<td>Cu(II) &amp; Bis-Cu(II) chelates</td>
<td>Lysozyme</td>
</tr>
<tr>
<td></td>
<td>egg white</td>
</tr>
<tr>
<td></td>
<td>92</td>
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<td>Various Proteins</td>
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<tr>
<td>Protease (IV)</td>
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<td>Protease (IV &amp; V)</td>
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<td>β-galactosidase (S-V)</td>
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<td>Asp-11 fusion</td>
<td>E. coli extract</td>
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<td>β-galactosidase</td>
<td>Fugal lactase</td>
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<tr>
<td></td>
<td>Alkaline protease</td>
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<tr>
<td></td>
<td>RNA polymerase I</td>
</tr>
<tr>
<td></td>
<td>RNA polymerase II</td>
</tr>
</tbody>
</table>

a Roman numerals indicate precipitate fraction. "S" preface indicates supernatant.
b Estimate based on assuming the same activity units/mass for all three enzymes.
c No mixtures of proteins were tested.
d Low yield due to impurities in chitosan. Yield improved to 100% after chitosan subjected to gel filtration twice.
e A homogeneous product was obtained. The separation factor approaches infinity by definition.
whereas the separation factor ($\alpha$) is defined as

$$\alpha = \frac{[\text{target protein}]_{\text{in precipitate}}}{[\text{other protein}]_{\text{in extract}}} \div \frac{[\text{other protein}]_{\text{in precipitate}}}{[\text{target protein}]_{\text{in extract}}}$$  \hspace{1cm} (2)

The third definition is different from $a$ in that the denominator is "in supernatant" instead of "in extract". This last factor is the least prevalent of the three and will not be used here primarily since it is only a variant of $\alpha$. The purification factor is merely a representation of the increase in specific activity of the target protein compared to that in the original extract. The separation factor differs from the purification factor in that it is a measure of the increase in the ratio, not percentage, of target protein to contaminant proteins upon precipitation. A purification or separation factor greater than unity indicates an enrichment of the target protein in the precipitate. The separation factor is more sensitive to the initial percentage of the target protein in relation to other proteins, as well as to the final purity achieved by the precipitation. For instance, in Table 1, the data for CMC precipitation of lysozyme from a mixture containing an equal amount of ovalbumin shows $\gamma = 1.84$ and $\alpha = 11.2$. For this lysozyme-rich starting material, $\gamma$ does not give a sensitive indication of the excellent selectivity achieved (perfect separation would give $\gamma = 2$). However, separation factors cannot always be calculated from literature sources, since data are often given only in terms of specific activity of the target protein. When available, both purification and separation factors will be used here.

Several factors should be taken into consideration when looking at the precipitation results in Table 1. First, the starting materials for the different precipitations varied widely. They include such solutions as artificial mixtures of
proteins, bacterial and yeast homogenates, and filtrates of yeast cultures. Crude extracts have been centrifuged to remove cell debris, and may have undergone further processing before use in precipitation. The initial and final percentage of the target protein in relation to the total protein vary from less than 0.1% to greater than 40%. Second, neither α or γ takes into account the large concentration of the product which occurs through precipitation, nor does either account for the separation from nucleic acids, lipids, etc., whose concentrations also vary widely in the initial mixtures. Both are only measures of the capability to perform the usually more difficult task of fractionating the proteins. Third, factors such as precipitation kinetics and entrainment may well have affected these results. In other words, the comparisons are not those of equilibrium processes. Such factors can lead to a "black art" perception of precipitation which deters its use. Finally, most of the data were published because by some measure the separation was successful. Only a few of the papers were reporting both successes and failures of the method.

For the purpose of narrowing this review to "selective" precipitation we will focus on only those precipitation agents demonstrating purification factors greater than 5 from crude extracts. A look at Table 1 shows those to include protein-binding dyes, macroligands, and polyelectrolytes. Despite the lack of data on metal ions, these will be included as well. Examples of several "nonselective precipitants" have been included on Table 1 for comparison. More information on the other agents may be found elsewhere. Protein-binding dyes and metal ions have been classified as macroligands, yet are considered as separate entities here due to their comparative chemical and biological stability, relative low cost, and differences in precipitating mechanisms.
Each of these "selective" methods shares the characteristic that precipitant and protein bind and it is the resulting complex that precipitates. Dye-binding associations are attributed both to electrostatic associations between charged dyes and charged regions of the protein or to the binding of a dye to a cofactor or substrate binding site on the protein. To be used as a precipitant, a dye must be capable of forming at least two associations per dye molecule. Metal ion affinity precipitation takes advantage of the interaction between divalent metal ions, such as Co\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), and Ni\(^{2+}\), and strong electron donor groups on the proteins. Macroligand affinity precipitation differs in that a biospecific ligand interacts with the target protein at its binding site(s). Polyelectrolyte precipitation is based on the ionic interactions between the polyelectrolyte and the proteins.

These precipitants all have their counterparts in adsorption and chromatography. Chromatographic techniques have, however, several limitations: (i) resistance to mass transfer through diffusional limitations and steric hindrance in the association step lead to slow binding and low available capacity; (ii) treatment of viscous or particulate matter can cause plugging and results in high pressure drops which limit flow rates; and (iii) the limits of scaling up due to bead deformation with higher pressure drops. Precipitation techniques overcome these limitations and furthermore have the advantages of high throughput and continuous operation. These advantages must be weighed against chromatography's advantages of multiple stages and insoluble separating agent. A comparison of purification factors obtainable by different separation methods was made recently by Bonnerjea et al. An expanded version of their findings can be seen in Figure 1. Their survey showed the typically low purification factors expected for precipitation. However, ammonium sulfate
Figure 1. Comparison of average and maximum purification factors obtainable by various separation methods (modified from Bonnerjea et al.²)
precipitation predominated in that survey. The higher purification factors seen with "selective precipitation" represents the data from Table 1. While they still fall short of affinity adsorption, the values do approach those of other chromatographic methods. For perspective we have also included overall purification factors for 2-4 stages of aqueous phase partitioning representing 14 cases reported by Kroner et al.17 One should also keep in mind that as an early step precipitation is typically used in the presence of a greater number of interfering substances than would have been present in the chromatographic steps.

In choosing a precipitation strategy, factors other than the choice of precipitant must be taken into consideration. Strategies to enhance precipitation include the manner in which the precipitant is added to the process stream and the environment in which precipitation occurs. Genetic engineering can be used to enhance the separation of the targeted protein through the fusion of peptides which confer characteristics enabling easier separation. And finally, the isolation of the product after precipitation has to be taken into account. These factors will be discussed in relation to general precipitation strategies and their application.
CHOICE OF PRECIPITANT

Binding and Selectivity

The binding between the precipitant and protein determines the selectivity of these methods. Higher specificity in binding results in greater purification of the desired product. The choice of precipitant must therefore be made so as to take advantage of any specific binding characteristics of the targeted protein.

Affinity interactions

Affinity macroligands and protein-binding dyes selectively bind proteins through strong biospecific associations between the protein and its ligands.\textsuperscript{18-20} The types of ligands available include substrates, coenzymes, immunoligands, etc. The strength of binding between the protein and the ligand is typically very strong, with $K_m$ values typically ranging from $10^{-4}$ to $10^{-10}$.\textsuperscript{21} Binding is relatively insensitive to solution conditions such as pH, ionic strength and protein concentration, as long as the structural integrity of the protein is not impaired. The presence of competitive ligands can greatly decrease or reverse binding.

Divalent metal-ion precipitants specifically bind to surface-accessible strong electron donor groups on the protein. Of the various amino acids comprising proteins, histidine, cysteine, tryptophan, and arginine possess such groups.\textsuperscript{22} Fractionation results from the variation in distribution of such groups on the surface of the protein.

Polyelectrolytes

The term polyelectrolytes will be used to indicate water-soluble polymers with a regular distribution of ionizable groups resulting in an expanded random coil conformation. The expansion of coil dimensions depends on chain flexibility and charge density; for weakly ionizable groups, the latter will change with degree
of dissociation and hence the pH. Examples of polyanions are poly(acrylic acid) and carboxymethyl cellulose; polyethylenimine is a polycation. Such molecules can complex with oppositely charged molecules to form species no longer stabilized by charge repulsion, solvation, etc. Precipitation is the result. Since polyelectrolytes are also used as flocculants (via surface-binding to aggregate particulates) in removal of suspended solids, their presence can additionally affect the character of the precipitate by this role.23

The binding of a protein to a polyelectrolyte is believed to be dependent on electrostatic, hydrophobic, and hydrogen bonding interactions between the protein and polymer.24-26 Electrostatic interactions are presumed to be the dominant forces. The presumption is supported by the facts that only proteins possessing a charge opposite the polyelectrolyte are precipitated, that highly charged proteins are selectively precipitated, and that the extent of precipitation decreases as the ionic strength is increased.27 Further support comes from the observation that the complexation of proteins with strongly ionizable polyelectrolytes follows a stoichiometric relationship when performed under conditions in which the acidic or basic groups of the protein are completely dissociated.28,29

For guidance in use of such precipitants it is worth considering these materials as soluble ion exchange resins (since such materials are essentially just crosslinked polyelectrolytes). On that basis precipitation would be expected to be most effective at low ionic strength and at a pH where protein and polyelectrolyte have opposite charge. In this case it is the complex, including precipitating agent, that precipitates. Hence the stoichiometry of the association will play a major role in determining required polyelectrolyte levels. The greater the polymer charge
density, the greater the capacity for protein binding. Experimental studies have demonstrated the role of charge equivalence.\textsuperscript{28-30}

In addition to considering binding capacity, one must consider the strength of the binding. This second factor will govern efficiency of removal and the selectivity of removal in mixtures. The analysis of Morrow, Carbonell, and McKoy\textsuperscript{31} for partitioning of proteins on ion exchangers gives some guidance for the basis of separation. Their results are based on a consideration of electrostatic and hydrophobic forces. The result is a partition coefficient dependent on the sum of those two interactions

\begin{equation}
\log \left( \frac{K_c}{K} \right) = \frac{-2AZ_pZ_bI^{1/2}}{1+BI^{1/2}} + CI
\end{equation}

where \( K_c \) is the concentration equilibrium dissociation constant for a complex of protein and binding sites, \( K \) is the true (activity) equilibrium constant, \( Z_p \) is the charge on the protein, \( Z_b \) is the charge on the binding site, \( I \) is the ionic strength, and the remainder are empirical constants. The two terms on the right account for electrostatic and hydrophobic interactions. Here the protein will bind to the polyelectrolyte.

Equation 3 indicates that the net charge on the protein will be a primary basis on which separation is made. A polyelectrolyte with high charge density may ensure that this is the basis of separation by affording little opportunity for the hydrophobic interactions to become significant. Here one would want to operate away from the isoelectric point of the protein. The equation also provides for disruption of binding by increasing ionic strength.
A model specifically addressing polyelectrolyte precipitation has been developed by Clark and Glatz.\textsuperscript{32} Their model assumes multi-site, cooperative binding. Cooperative binding accounts for the influence of bound ligands on subsequent binding. The enhancement or deterrent of further binding is termed positive or negative cooperativity, respectively.\textsuperscript{32-34} Cooperativity is an effect often observed in biological systems. A modification of the Debye-Huckel theory is used in the theory to account for the electrostatic effects responsible for negatively cooperative binding. The model showed the effects of protein charge and ionic strength on the precipitation of ovalbumin and lysozyme with CMC.

\textbf{Solubility and Precipitation}

A typical globular protein presents to the solvent a surface consisting of positive and negative charged regions, polar, yet uncharged, hydrophilic regions, and nonpolar, hydrophobic regions. Proteins are of high molecular weight (typically 10 000 to 500 000 Da), yet compact, particularly in comparison with other high molecular weight components such as nucleic acids and polysaccharides. Different proteins exhibit different relative proportions of the various surface types. The complex interactions between the protein surface and surrounding solvent determine the solubility. The protein remains in solution when it is thermodynamically more favorable to be surrounded by solvent than it is to be aggregated with other protein molecules in a solid phase.

Of the specific precipitants discussed here, all except heterogeneous macroligands result in the formation of an insoluble complex upon mixing with the protein. This proceeds via the formation of an insoluble complex upon binding of the protein and the precipitant, rapid formation of a solid phase in the form of submicron primary particles, followed by larger-scale aggregation through the
shear-driven collision of primary particles and/or small aggregates with growing aggregates. Primary particle size typically increases with protein concentration; aggregate size increases with protein concentration subject to the limits of breakage.

**Macroligands**

The various types of affinity precipitants can be classified as homogeneous and heterogeneous macroligands. Homogeneous macroligands are ligands bound by a linker to other ligands or several bound to a macromolecule. Binding of the macroligand to the protein brings about both the selective fractionation of the protein, as well as the formation of large, crosslinked aggregates. The aggregates grow until they become large enough that they are no longer soluble and precipitate. For the crosslinking to occur, the target protein must bind at least two ligands (i.e. bifunctional).

Figure 2 depicts the mechanism of precipitation for homogeneous macroligands. The mechanism also applies to other bifunctional affinity precipitants, which include metal ions and protein-binding dyes. The crosslinking of the ligands and the proteins leads to the formation of a large, insoluble complex. An early example of bifunctional affinity precipitation used bis-NAD derivatives to precipitate dehydrogenase enzymes. The NAD ligands were attached to the ends of a spacer molecule (linker), thus forming the macroligand.

The ligands of heterogeneous macroligands are responsible only for the selective fractionation upon binding. The nature of the carrier molecule determines the conditions under which the macroligand-protein complex will precipitate. Typically, precipitation is effected through changes in the pH, ionic strength, or temperature of the solvent. The addition of a secondary precipitant which selectively crosslinks the macroligand-protein complex is another option. In this
Figure 2. Affinity precipitation using homogeneous bifunctional macroligands (after Flygare et al.36)
case, the result is the formation of a large, insoluble complex similar to that depicted in Figure 2.

Protein-binding dyes

The formation of precipitates upon the complexation of protein-binding dyes with proteins is very similar in manner to that of homogeneous bifunctional macroligands. The protein-binding dye also functions as a bifunctional precipitant, complexing with two other proteins. The result is the formation of a large, crosslinked structure as is depicted in Figure 2.

Metal ions

The complexation between divalent metal ions and the strong electron donor groups of proteins results in the formation of large, crosslinked aggregates (Figure 2).^{38,39} When the aggregates have grown sufficiently large, they become insoluble and precipitate. Because one ion can chelate with multiple donor groups, the precipitant can be in the form of either the simple ion or that of a bis-chelate. A bis-chelate consists of two metal ions attached to the ends of a spacer molecule.

Polyelectrolytes

The binding mechanism for the final aggregation of primary particles into flocs has been variously attributed to the mechanisms of patching^{40-44}, charge neutralization^{45,46}, or bridging^{47-49}. Figure 3 shows a schematic portraying these steps for the case of polyelectrolyte precipitation. The patching model assumes that parts of the primary particles remain charged upon formation. The positive and negative patches on the surfaces of the primary particles can then strongly interact to form flocs. In the charge neutralization model, the net charge on the primary particle is assumed to be nearly or completely neutralized, thus reducing protein solubility as well as the electrical repulsion between protein molecules. Polymer
Figure 3. Schematic of the precipitation process as proposed by Clark and Glatz^43
attachment between primary particles and aggregates to form flocs is the basis of the bridging model.
CONSIDERATIONS OTHER THAN SELECTIVITY

Clearly, the proper choice of precipitating agent is important to achieving high selectivity. Whatever the choice, there remain several other strategies with which selectivity may simultaneously be pursued. One must tend to the physical aspects of precipitator operation. One can consider the influence of upstream operations and potential pretreatments on the composition of the protein-containing stream. Going even further upstream, one can consider genetic engineering as a means of modifying the protein to simplify the separation task. And finally, since one has added a material separating agent, one is confronted with the task of removing the precipitant.

Physical Aspects of Precipitation

Protein solubility generally depends on not only on concentrations of the protein and precipitant, but also on such factors as pH, ionic strength, temperature, and the concentrations of other components of the mixture. Even accounting for all of these factors would not be sufficient to determine behavior, however, as the results frequently depend on more than thermodynamics. Of importance are such considerations as the inlet concentration and rate of addition of the precipitant, the mechanism of contacting, the duration and level of mixing, and the final recovery of the precipitate.

Addition of precipitant

Though not among our list of "selective" methods, salting out provides an excellent example of the potentially non-equilibrium nature of solubility behavior. Figure 4 shows a series of salting out curves for fumarase obtained by adding ammonium sulfate in a variety of ways. Clearly different results are obtained
Figure 4. Effect of contacting procedure on final equilibrium precipitation behavior of fumarase. ▼, ● - batch contacting; □, ▲ - continuous contacting, 16.9 minutes total residence time; ▼ - ammonium sulfate solid; ●, □, ▲ - saturated ammonium sulfate solution. Other conditions were: ▼, □, ▲ - pH 5.9, 40 mg/ml initial protein, 8°C; ● - pH 5.7, 35 mg/ml initial protein, 6°C (from Foster et al.).
when the salt is added as a solid than as a saturated liquid. Yet another factor is seen to be important in the design of the precipitator.

The difficulty revealed in this example is that the kinetics of reagent dispersion are not sufficiently rapid to ensure that all regions of the vessel are of uniform concentration during the time that the precipitate is forming. The penalty is over- and coprecipitation. Various schemes have been proposed to achieve homogenous nucleation of precipitates. Long ago Meekin\(^1\) added ethanol by dialysis and this was repeated for acid addition in isoelectric precipitation by Fisher and Glatz.\(^2\) Other strategies include the circulation of a protein stream from a reservoir through a flow loop where precipitant is gradually introduced at a static mixer followed by recycle of the stream to the reservoir.\(^3\) Workers at Merck\(^4\) have used a continuous variation of this, joining antibiotic and precipitant in a jet mixer. In both cases, more consistent results and/or a better quality precipitate has been reported.

Mixing

The final state of the precipitate is dependent on the duration and level of mixing to which the suspension is exposed. Foster, Dunnill, and Lilly\(^5\) reported on the changes in precipitate composition that resulted from up to several hours of aging of ammonium sulfate precipitates of yeast enzymes. A series of papers on changes in the physical characteristics of protein precipitates with exposure to shear has been reviewed by Bell, Hoare, and Dunnill.\(^6\) Aging increases the strength of the precipitate (as evidenced by resistance to high shear breakup) up to a Camp number (the dimensionless product of shear rate and mixing time) of \(10^5\).\(^7\) The increased strength results from restructuring of the floc and the same restructuring
may affect the amount of entrained solution and behavior during solid/liquid separation steps.

The initial formation of the floc structure is also controlled by mixing as floc growth is the result of hydrodynamically driven collisions of primary particles and small aggregates with the growing flocs.\textsuperscript{57} For most precipitations the initial formation of primary particles occurs on the order of seconds and even floc growth is largely complete within tens of seconds. In contrast, completion of precipitation using a homogeneous macroligand to recover a dilute protein from a complex mixture has been reported to take several hours.\textsuperscript{58} And while polyelectrolyte precipitation proceeds with typical rapidity at optimal pH conditions, the rate was observed to be much slower two pH units away from the optimum.\textsuperscript{59}

**Recovery of the precipitate**

After the precipitate is formed, it must be separated from the liquid and the protein subsequently separated from the precipitant. To do the former requires an operation such as settling, flotation, centrifugation, traditional filtration, or cross-flow membrane filtration. For most of these methods, particle size becomes the determining factor in the ease and speed of the process. Hence, the formation of large flocs is desirable. For those techniques involving filtration, an additional problem is the high cake resistance of the typically compressible protein precipitates. The use of filter aids and/or cross-flow operation are generally necessary to overcome this problem. Further information on the nature of aggregate strength and its consequences can be found elsewhere.\textsuperscript{60}

**Nature of the Extract**

Obtaining a pure product is particularly complicated when one considers the fact that cells consist of a complex mixture of many different components, including
cell walls (lipid bilayers), ribosomes, nucleic acids, and proteins. To complicate matters further, even in such simple cells as bacteria there are on the order of 1000 different proteins.

The demands on product purification are dependent on the fermentation process. If the product is secreted by the microorganism, the product will be fairly dilute in the broth, yet relatively pure. On the other hand, if the product is contained within the microorganism, either in the periplasmic space or in the cytosol, the cells must be harvested and subsequently disrupted to recover the product, which will be concentrated, yet relatively impure. Dilution often improves product recovery from streams (such as cheese whey) which possess prohibiting concentrations of interfering components (such as salts). To avoid unnecessary product losses, conditions during fermentation and purification must be adjusted so as to avoid denaturation or proteolytic degradation of the product.

Components such as cell debris and nucleic acids can interfere with, and must often be removed prior to, protein precipitation. The removal of cell debris prior to the precipitation of crude extracts is necessary to avoid contamination of the precipitate, and thus the product, by the cell debris. Polyelectrolytes have been used to combine the removal of nucleic acids and cell debris in one step. Evidence as to the improvement in selectivity upon removal of nucleic acids can be found in the precipitation of genetically modified β-galactosidase with PEI.

**Impact of Genetic Engineering**

Genetic engineering can be used to produce greater product yields and to enhance the separation characteristics of targeted proteins. Through increased expression levels (up to 45% of total protein in the cell) and choice of
microorganism, recombinant organisms can now create mass quantities of products which were once very difficult to extract, and thus very rare and expensive.\textsuperscript{65,72}

Enhancing the separation of a protein has typically been done by genetically fusing a polypeptide conferring a basis for recovery to the terminus of the desired protein. Such genetic fusions are referred to as purification fusions or "tails". The tails have included charged amino acids for recovery by ion exchange, multiple histidines for recovery by metal ion affinity, and whole proteins for recovery by ligand affinity.\textsuperscript{73-78} Many fusion tails do not interfere with the activity of the enzyme and may even offer protection from proteolytic degradation.\textsuperscript{79-84} Thus far the tails have primarily served for adsorptive binding, but the technology is readily adaptable to application in precipitation.

For example, ion exchange chromatography has previously been shown to enhance the recovery of a genetically modified small protein, \(\beta\)-urogastrone, containing positively charged fusion tails.\textsuperscript{85,86} Similarly, negatively charged aspartic acid tailed \(\beta\)-galactosidase fusions showed improved separation behavior relative to wild-type \(\beta\)-galactosidase on an ion exchange column.\textsuperscript{87,88} Precipitation of \(\beta\)-galactosidase fused with a tail of 11 aspartic acid residues with polyethyleneimine at a precipitant:protein ratio of 0.01 gg\(^{-1}\) resulted in a separation factor of 7.9. This contrasts with the unmodified protein which was not enriched in the precipitate formed under the same conditions.\textsuperscript{69} The fusion did not interfere with the enzymatic activity. Given the size of and large number of charged groups on \(\beta\)-galactosidase, application of this strategy to smaller proteins may well give even better results.
Removal of Precipitant

The separation of the protein from the precipitant is necessary most of the time. Economics often require the recovery and reuse of the precipitant. Also of concern is whether the precipitants are acceptable agents for bioprocessing. Some precipitating agents are not approved for use with food or pharmaceutical products. Other agents may interfere with subsequent processing steps. Typically, the precipitate is washed to remove any non-specifically included substances. It is then resuspended in a buffer in which the precipitating agent and the protein dissociate. This buffer is preferably one which can be directly used in subsequent processing steps. In the case of heterogeneous macroligand affinity precipitation, the macroligand can be designed so that it remains insoluble upon dissociation from the protein. For cases in which both the precipitant and protein remain soluble, separation can be achieved using ultrafiltration or chromatographic methods. If the protein and precipitant are of substantially different size, ultrafiltration can be used to achieve quick separation. Ultrafiltration has the advantage of continuous operation. Chromatographic techniques offer a variety of bases upon which separation can be accomplished such as size, charge, and hydrophobicity.
USE OF SELECTIVE PRECIPITANTS

In addition to the separation factor, the following must be considered in choosing a precipitant for a specific separation task:

1) are traces of the soluble precipitant acceptable,
2) how can the soluble precipitant be separated from the target protein,
3) is the precipitant stable in the extract,
4) how much of the precipitant is needed and how critical is the dosage,
5) is the process sensitive to the values of pH, ionic strength, temperature, etc., and if so, to what extent?

Each of the selective precipitation methods has different answers to these questions. The following sections discuss these and other important aspects for each precipitating agent.

Affinity Macroligands

The basis of affinity macroligand precipitants lies in the attachment of a ligand to a soluble carrier. Binding of this macroligand to the target protein results in the formation of a complex which can be precipitated out of solution. Affinity precipitation can be divided into two categories corresponding to the nature of the precipitant: homogeneous macroligands and heterogeneous macroligands.

Homogeneous macroligands

Homogeneous bifunctional macroligands consist of two identical affinity ligands connected by a spacer; also termed bis-ligands. The first extensive studies of affinity precipitation used bis-NAD to precipitate multimeric dehydrogenases. For bis-ligand affinity precipitation to work, the following requirements have to be met:

1) the enzyme must have more than one binding affinity site,
2) the bifunctional ligand must have a strong affinity for the enzyme, and
3) the spacer which binds the ligands must be of sufficient length to bridge
   the distance between the binding sites on the enzymes, yet not so long as
   to bind two sites on the same protein.

The precipitant has the advantage of biospecificity, which can lead to very high
purifications. The conditions of precipitation are typically mild enough that neither
protein nor ligand is denatured. Disadvantages include the high cost and
susceptibility to proteolytic degradation of homogeneous macroligands. Recovery
of the macroligand is therefore very important.

Research using bis-biotinyl compounds to selectively and strongly bind
avidin displayed a marked effect of linker length upon the final structure of the
complex. Only one of the two biotin residues separated by
polymethylenediamine linkers of less than 14Å could bind to an avidin molecule,
whereas linker lengths exceeding 38Å could result in the reagent binding two
subunits of the same avidin molecule. Intermediate linker lengths were found to
form intermolecular complexes. Larsson and Mosbach studied the role of spacer
length using bis-NAD with spacer lengths of 7, 17, and 32 Å. A spacer length of
17 Å (using N₂,N₂' adipodihyrazido-bis(N⁶-carbonylmethyl-NAD)) was found to
be optimal in precipitations with various dehydrogenases. The selectivity and
effective strength of binding of the bis-NAD system were increased through ternary
complex formation by adding competitive inhibitors to the solution. For example,
the addition of the competitive inhibitors pyruvate or oxalate to precipitations
involving the tetrameric enzyme lactate dehydrogenase resulted in the formation of
strong ternary complexes.
The yield of the precipitation using bis-NAD macroligands was found to be dependent on the ratio of NAD ligands to enzyme subunits. An optimum would be expected at ratios near unity. At ratios higher than unity, a decrease in yield is expected since not all ends of the bis-ligands will be able to complex with enzyme subunits. At ratios lower than unity, not all enzymes will be bound.

Experimentation confirmed an optimum near unity for precipitations of LDH at a ratio of 1.25 NAD ligands:LDH subunits. Precipitations with oligomers possessing a greater number of subunits are affected to a lesser extent by deviations from a ratio of unity. The hexamer glutamate dehydrogenase yielded almost quantitative precipitation between ratios of 0.3 and 10. A problem which may be encountered with this method is the formation of linear polymers or dimeric complexes. The latter problem was encountered when attempting to precipitate liver alcohol dehydrogenase. Soluble complexes were formed which consisted of two enzymes bound by two bis-NAD molecules.

In order to observe selectivity in a mixture of enzymes, precipitation of LDH was performed on ox heart crude extract. The precipitation resulted in a purification factor of 40 and a yield of 91% for greater than 95% purity. However, the time required for complete precipitation was relatively long at a minimum of two hours. Dissolution of the complex after centrifugation was accomplished by the addition of the competitive ligand NADH.

A more recent development in using homogeneous macroligands for affinity precipitation is the binding of multiple ligands to a polymer. Preliminary results using biocytin bound to dextran for the precipitation of avidin appear to be promising. Up to 90% of the avidin in solution could be bound under optimal conditions. The same conditions yielded only 3% precipitation when using...
lysozyme as a control. Optimum precipitation was obtained using low ionic strength and highly substituted, low molecular weight dextran.

**Heterogeneous macroligands**

The use of heterogeneous macroligands has several advantages over the use of bifunctional affinity ligands. First, rather than a sharp increase in yield at the required stoichiometric ratio of ligand to protein, the yield steadily increases with the amount of heterogeneous macroligand added, making it easier to adapt the precipitation to changes in protein concentration. Second, precipitation is not coincident with complex formation. The mechanism by which precipitation occurs can be chosen independently from the binding step. The only restriction is that the conditions for precipitation do not also dissociate the protein-ligand complex. Subsequent separation of protein and macroligand is easiest if there are conditions where only the protein can be extracted from the precipitate. Thirdly, heterologous precipitants are not limited to multimeric proteins. However, the ligand moieties still suffer from their susceptibility to proteolytic degradation as well as their high cost. Protein activity retention has been good for the cases reviewed here.

Schneider et al. first demonstrated this technique. They used a terpolymer of N-acrylol-p-aminobenzamidine, acrylamide, and N-acryloyl-p-aminobenzoic acid which is soluble at neutral pH but insoluble in the acid form at low pH. The p-aminobenzamidine moiety acts as the affinity ligand for trypsin. After binding, precipitation was induced by lowering the pH to 4.0. Dissociation of the complex was accomplished by further lowering the pH to 2.0. Application of the procedure to beef pancreas extract resulted in a trypsin yield of 79% and a separation factor of 38 for 90% purity. The remaining protein in the precipitate consisted essentially of the very similar protein chymotrypsin, yet only 6.3% of the original chymotrypsin
was present in the precipitate. After re-using the macroligand up to eight times, the separation factor decreased only to 31, giving 84% pure trypsin at a yield of 76%. The average loss of the macroligand was given as 1% per cycle; 93% of the macroligand remained after 8 cycles.

Other pH dependent heterogeneous macroligands include those based on chitosan\(^{94,95}\) (used as the backbone in the recovery of trypsin and WGA) and hydroxypropyl methylcellulose acetate succinate (AS)\(^{96}\) (used for recovery of protein A). Chitosan, a partly deacetylated chitin (obtainable from shrimp and crab shells), is rich in the polycationic repeat unit \(N\)-acetyl-D-glucosamine and is insoluble above pH 6.5. The macroligand allowed for dissolution and protein dissociation at pH 2.5. Trypsin was separated from the soluble chitosan macroligand by gel permeation chromatography.\(^{94}\) In the affinity precipitation of WGA with chitosan,\(^{95}\) dispersed gas flotation was used in place of centrifugation for precipitate isolation. AS, insoluble below pH 4.5, was coupled to IgG to bind protein A.\(^{96}\) After precipitation at pH 4.5, protein A could be extracted from the precipitate at pH 2.5. Recyclability of the macroligand was demonstrated over four cycles with an average yield of 91% and a separation factor of 67.

Temperature and ionic strength have been used for solubility control with NIPAM-GMA (a copolymer of \(N\)-isopropyl acrylamide (NIPAM) and glycidyl methacrylate (GMA)) as the carrier molecule for the trypsin-binding ligand PAB. Raising the temperature above 34°C gave macroligand and trypsin recoveries of 95% and 82%, respectively, from trypsin/chymotrypsin mixtures, with only 2% of the chymotrypsin coprecipitating.\(^{91}\) This backbone was also used as a carrier for IgG to recover protein A conjugates.
In addition to using pH, temperature, or ionic strength variations to induce precipitation, a method has been recently introduced which uses a biospecific crosslinking agent to induce precipitation.\textsuperscript{16} The multivalent lectin concanavalin A (Con A) was used as the agent to biospecifically crosslink the Blue Dextran macroligand/LDH complex by binding glucose residues and thereby effect precipitation. The LDH was bound to the Cibacron blue residues. While the precipitation was independent of the ratio of Blue Dextran to LDH, the ratio of Con A to Blue Dextran had to be optimized. Recovery of the target enzyme from the complex is complicated by entrapment of the enzyme in the complex and by the presence of Con A. These workers removed the Con A by binding to DEAE-Trisacryl gel and the blue dextran by gel filtration. A similar approach was used to recover IgG from serum by binding to protein A immobilized on galactomannan followed by precipitation through non-covalently crosslinking with KBO\textsubscript{4}.\textsuperscript{97} Dissociation of IgG was accomplished by adding KSCN. Subsequently, the macroligand-borax complex could be dissociated by lowering the pH.

A novel approach has recently been demonstrated using affinity surfactants to specifically precipitate multimeric proteins.\textsuperscript{98} The macroligand consists of an affinity ligand which has been covalently attached to the polar head group of the surfactant. The study focused on the use of dimyristoylphosphatidylethanolamine-biotin (DMPE-B) to selectively precipitate the egg white protein avidin. Precipitation was thought to result from the binding of a single avidin to four macroligands. The hydrophobic tails then interact to form a network similar to that proposed for homogeneous bis-ligands (see Figure 2). Precipitations of CMC-pretreated (to remove hydrophobic and aggregating impurities) hen egg whites resulted in 91\% of the avidin being removed. Greater than 80\% of the lysozyme
activity was retained after centrifugation, resolubilization, dissociation of the complex by denaturation, ultrafiltration to remove DMPE-B, and renaturation. The corresponding separation factor achieved by the overall process is 110. Among the advantages of this process over other affinity precipitation methods are that the synthesis of the macroligand is generally simpler and cheaper, and that the phospholipid does not contain any charges which could lead to non-specific interactions.

Protein-binding dyes

Triazine dyes have become widely used in protein purification. When attached to solid supports, triazine dyes exhibit high protein-binding capacities towards some proteins and the bound protein can easily be dissociated at mild conditions.99 The binding is thought to be largely ionic and the resulting protein-dye complex is more hydrophobic and can precipitate.19 An example of such a dye precipitant is Rivanol, an organic cation which has been used to purify serum proteins.14

Recently, triazine dyes which specifically bind certain classes of proteins apparently through affinity interactions have been described. Bis-ligand affinity precipitants have been constructed by covalently linking two of these dye molecules (ie. Cibacron Blue) via a spacer molecule.100,101 However, attempts to use poly(Cibacron Blue) conjugates to precipitate LDH were unsuccessful.102 The use of protein-binding dyes rather than biological ligands for use in affinity macroligand precipitations has the advantage of lower cost and stability when exposed to crude cell lysates.

Among the proteins bound by Cibacron Blue are the NAD-dependent dehydrogenases. The dye has been derivatized to increase its specificity towards
the dehydrogenases.\textsuperscript{20,103,104} Earlier experiments with bis-(Cibacron Blue) derivatives showed limited selectivity towards the cofactor-dependent enzymes.\textsuperscript{100,101} However, a simple methoxylated derivative of the \textit{p}-sulphonate isomer of Cibacron Blue F3G-A resulted in specific precipitation of LDH from rabbit muscle crude extract.\textsuperscript{103}

The precipitation is believed to be the result of crosslinking the LDH molecules with the dye to form a large insoluble complex. The dye acts as the functional analogue of bis-NAD, with the anthraquinone moiety serving as one binding site, the methoxytriazinyl ring and terminal \textit{p}-aminobezenesulfonate ring serving as the other binding site, and the central \textit{p}-phenylenediaminesulfonate ring acting as the linker (see Figure 5). Support for this mechanism comes from the fact that rapid dissociation is achieved by the addition of relatively low concentrations of competitive ligands such as NADH. Furthermore, the maximum precipitation displays the optimal molar ratio of enzyme subunit:dye of 2:1, which is expected for such a mechanism. The entire cycle time for preparative precipitation, including tissue homogenization, DEAE-Sepharose pretreatment, enzyme precipitation and dissolution, and separation of the enzyme and precipitant via gel chromatography was approximately 3 hours.\textsuperscript{104}

"Affinity constrained precipitation" is an interesting variation in this precipitation strategy which operates by forming a soluble affinity complex with the target protein under conditions which precipitate other proteins. Johansson and Joelssson\textsuperscript{105} used high concentrations (12.5\%\textit{v/v}) of PEG to precipitate undesirable proteins while a small fraction of PEG with attached dye moieties formed soluble complexes with dye-binding proteins. The method was used to purify glucose-6-
Figure 5. Structure of the methoxylated p-sulphonate isomer of Cibacron Blue F3G-A (after Pearson et al.\textsuperscript{104})
phosphate dehydrogenase and 3-phosphoglycerate kinase from a crude extract of baker's yeast. The overall yield was 93% for a 3.4-fold purification.

**Metal Ions**

Experimentation using metal affinity precipitations has been limited thus far to demonstrating the use of bis-copper chelates to crosslink proteins containing multiple surface accessible histidine residues.\(^{38,39}\) No data currently exists for precipitations from crude extracts. Two different bis-chelates were used in these studies: PEG-Cu(II), composed of cupric cations chelated by molecules of iminodiacetic acid and immobilized on each end of polyethylene glycol (PEG-2000); and \(\text{Cu(II)}_2\text{EGTA}\), composed of two cupric cations chelated by a molecule of ethylene glycol bis(β-aminoethyl ether)\(N,N'\)-tetraacetic acid (EGTA). The bis-copper chelates were shown to be effective in precipitating proteins such as human hemoglobin and sperm whale myoglobin which have multiple surface-accessible histidine residues (26 and 6 respectively). Precipitations with \(\text{Cu(II)}\text{EGTA}\) showed that human hemoglobin precipitated to 100% at a copper to surface accessible histidine ratio of unity, whereas at this concentration, sperm whale myoglobin was precipitated to less than 10%. A protein which contains only one surface-accessible histidine, horse heart cytochrome c, could not be precipitated even when large quantities of bis-chelates were added. The higher molecular weight PEG-Cu(II) was shown to be a more effective precipitant on a molar basis. Precipitations carried out using an excess of bis-chelate revealed a 1:1 stoichiometric ratio of copper:surface-accessible histidine in the precipitate. Of importance in the design of such bis-chelates is that the metal ions bind strongly to the carrier so that they will not be lost under the conditions necessary to dissociate the protein from the complex.
The use of recombinant technology should lend itself readily to improving the separation characteristics of a protein via metal-ion affinity precipitations. Possibilities for application in precipitation can be seen in recent articles on the metal-ion affinity chromatography of recombinant proteins.\textsuperscript{22,106,107} For example, the addition of two histidine peptides to the carboxyl end of mouse dihydrofolate reductase resulted in greatly enhanced recovery of the enzyme on an immobilized nickel column.\textsuperscript{107} Fusions of up to six histidine residues at either end of the enzyme displayed an increasing affinity for the column with increasing residue number.

**Polyelectrolytes**

For polyelectrolyte precipitation, the net effect of the electrostatic repulsion between protein molecules is minimized upon complexation between the polyelectrolyte and protein.\textsuperscript{108} Advantages of the method include high removal efficiencies and retention of enzymatic activity.\textsuperscript{24,27-29,109,110} High removal efficiencies result partly from the fact that polyelectrolytes can disrupt already existing associations, or be performed at a pH which does so. Very low amounts of polyelectrolyte (0.05-0.10\% wt/vol) are required and the fractionation potential is good.\textsuperscript{13} Since the ionic moieties on polyelectrolytes can range from strong acids to strong bases, precipitants are available over a wide pH range. Yield and floc characteristics are dependent on the polyelectrolyte/protein ratio. Reclamation and recycling of the polymers can be accomplished.\textsuperscript{111,112} Separation of the polyelectrolyte from the protein can be accomplished on the basis of charge, size, or solubility. Some of the polyelectrolytes have also been approved for ingestion.

The studies of polyelectrolyte precipitation include the fractionation of artificial mixtures of proteins\textsuperscript{24,52,61,113,114}, nucleic acids\textsuperscript{63,68,115}, the recovery of whey proteins\textsuperscript{114,116-118}, and the fractional recovery and isolation of serum
glycoproteins\textsuperscript{119}, recA protein\textsuperscript{120}, and viral proteins\textsuperscript{115}. Factors which have been found to affect polyelectrolyte precipitation include system pH and ionic strength; the molecular weight, charge density, dosage, and type (ionic group and backbone) of the polyelectrolyte; and the size and surface characteristics of the protein.

The degree to which various proteins will interact with a polyelectrolyte under identical solution conditions depends on the surface characteristics of the individual proteins. Both the number and distribution of charged sites on the protein surface determine the strength of the protein-polyelectrolyte complex.\textsuperscript{30} Only proteins possessing a charge opposite to that of the polyelectrolyte are precipitated, and those of higher charge density are precipitated preferentially.\textsuperscript{27}

Several authors have demonstrated this effect by precipitating artificial mixtures of proteins with polyelectrolytes. Sternberg and Hershberger\textsuperscript{24} fractionated a mixture of four proteins with PAA, whereas Clark and Glatz\textsuperscript{27} precipitated a binary mixture of lysozyme and ovalbumin with the polyanion CMC. In the latter experiment, complete separation between the two proteins was obtained at neutral pH values where only the lysozyme possessed a net positive charge.

The selection of system pH and ionic strength are critical since polyelectrolyte precipitation is a charge-based separation. Several authors\textsuperscript{26,27,30,109,114,117,118} have demonstrated that increasing the ionic strength leads to a decrease in the separation factor and higher polymer dosage requirements. Furthermore, Hill and Zadow\textsuperscript{118} found that this effect of ionic strength on precipitation was dependent upon the polyelectrolyte used to effect precipitation. Increased ionic strength reduces the effect of the pH on precipitation behavior and can even enhance fractionation selectivity if the target protein is highly charged.\textsuperscript{27} The optimum pH for a given process will be dependent on the
particular protein as well as the associated polyelectrolyte, since a change in pH affects the protein charge distribution and the net charge of both. These solution characteristics may also be important in that they may affect the flexibility of the polyelectrolyte and thereby influence precipitation through steric factors.

Protein recovery levels have been found to increase upon increasing charge density, molecular weight, and dosage of the polyelectrolyte. These same authors have emphasized the need for careful control of the polyelectrolyte dosage, which has been found to be dependent on the charge density and molecular weight of the polyelectrolyte, its degree of ionization, and on the target protein. Addition of excess polyelectrolyte can result in reduced protein recovery, an effect which may be less severe for proteins and polyelectrolytes of high charge density and polyelectrolytes of high molecular weight. Hidalgo and Hansen have proposed that the loss from resolubilization is due to a redistribution of protein in the complex as more polyelectrolyte becomes available. The result is a change in the stoichiometry of the complex. Support for this theory comes from measurements of the zeta potentials of aggregates made by Clark and Glatz. They found that the zeta potential decreased as the polymer dosage increased, indicating that the proportion of polymer in the complex increases with increasing dosage.

Precipitations of enzymes from various crude extracts have yielded impressive results. A sampling of results for precipitations involving various polyelectrolytes, including PAA, CMC, and PEI, as well as two heteropolyacids (WBA and WPA) can be seen in Table 1. Typical yields exceed 90% for purification factors as high as 29.
Experimental protocol typical of polyelectrolyte precipitation can be seen in the precipitation of lysozyme from egg white using PAA. The precipitation was carried out at room temperature and required only 5 minutes. Dissolution of the recovered precipitate was accomplished by raising the ionic strength and the pH of the system. Due to the difference in sizes between lysozyme and PAA, ultrafiltration was used to remove the polyelectrolyte and obtain a permeate containing purified lysozyme.

Another polyelectrolyte commonly used in precipitation is the cationic PEI. A problem encountered with the use of PEI to effect precipitation from cell homogenates is the associated interference from binding of nucleic acids to PEI. In fact, it has been shown that selective removal of nucleic acids from crude extracts can be accomplished by PEI precipitation without much loss of proteins if the ionic strength is above 1.0M at neutral pH. A high charge density on the protein can partly offset this interference from nucleic acids. When peptides of 5 and 11 aspartic acid residues were genetically fused to the carboxyl end of β-galactosidase from E. coli, precipitation with poly(ethyleneimine) was enhanced. Precipitation of crude cell extracts revealed that the longer tailed enzyme could be selectively separated from solution at high yield (85%) with a separation factor of 2.2, whereas the shorter tailed version and native enzyme could not. Nonetheless, a pretreatment of the extract to remove the nucleic acids did lead to still higher selectivity. Additional examples of selective precipitation using PEI can be found in the review by Jendrisak.

Sternberg demonstrated the use of heteropolyacids to selectively precipitate proteins from culture supernatants. Heteropolyacids are different in nature from the polyelectrolytes discussed above. The degree of polymerization of
heteropolyacids is dependent on the pH of the solution. Polymerization occurs in acidic solutions. Heteropolyacids are formed in solutions containing molybdate or tungsten and other oxo ions (e.g., $\text{PO}_4^{3-}$, $\text{SiO}_4^{4-}$) or metal ions. After recovery of the precipitate, the protein can be obtained by raising the pH whereupon the heteropolyacid depolymerizes, resulting in dissolution of the complex. The binding between the heteropoly acid and the protein was attributed primarily to ionic interactions, although a degree of affinity towards certain amino acids was found.
SUMMARY

The use of precipitation has been shown to be an efficient method for selective separation of proteins from crude biological mixtures. The precipitants which have demonstrated highest selectivity include polyelectrolytes, metal ions, protein-binding dyes, and biospecific affinity macroligands. Affinity macroligands selectively precipitate a target protein through biospecific binding to a ligand and can be classified as either homogeneous or heterogeneous, depending on how precipitation is induced. Heterogeneous macroligands allow for the properties which effect precipitation to be chosen independently of the protein-binding properties. Affinity macroligand precipitation can be extended to a wider range of proteins through genetic engineering of purification fusions. Major disadvantages of these precipitants include biodegradation and high cost. The three other precipitants overcome these disadvantages to a great extent, yet are generally not as specific towards the targeted protein. Polyelectrolytes effect separation on the basis of charge. The precipitant is relatively cheap, stable in crude biomixtures, and can be recycled. Their use can also be extended through genetic fusions. Literature concerning the other two methods is sparse. The affinity of metal ions towards histidine residues has been demonstrated in precipitation, yet wider applicability may be found by looking at current uses in chromatography. Such observation shows that proteins can be modified by the addition of metal ion affinity fusion peptides which should enhance their separation via precipitation. Selective binding of derivatised dyes to proteins has been shown to occur through affinity interactions. The precipitant is a less expensive version of the affinity macroligands and is stable in biological mixtures. Broad applicability is hindered by the limited variety of dyes which have been found to be selective.
Factors other than the choice of precipitant must also be taken into account in designing a precipitation process for maximum selectivity. Such factors include the manner in which the precipitant is introduced into the process stream, the environment in which precipitation will occur, the solids/liquids separation after precipitation, and the subsequent separation of the targeted protein from the precipitant. The optimization of each of these factors will vary depending on the precipitant to be used, as well as on the target protein. Special attention must be made in the design so as to minimize the losses of either precipitant or protein.

Given that these factors have been properly optimized, selective precipitants can achieve selective recovery of a target protein while at the same time effecting a substantial concentration. The power of using selective precipitants to achieve protein fractionation is evidenced by experiments where homogeneous protein products have been isolated in excess of 90% overall yield from crude biological mixtures.
REFERENCES


PAPER II: CHARACTERIZATION AND POLYLECTROLYTE PRECIPITATION OF β-GALACTOSIDASE CONTAINING GENETIC FUSIONS OF CHARGED POLYPEPTIDES
CHARACTERIZATION AND POLYELECTROLYTE PRECIPITATION OF β-GALACTOSIDASE CONTAINING GENETIC FUSIONS OF CHARGED POLYPEPTIDES

by

Mark Q. Niederauer¹,
Ilari Suominen²,
Malcolm A. Rougvie³,
Clark F. Ford²,
and Charles E. Glatz¹

¹Department of Chemical Engineering
²Department of Genetics
³Department of Biochemistry and Biophysics

Iowa State University
Ames, Iowa 50011

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ABSTRACT

Genetically engineered proteins were constructed through the addition of charged polypeptide fusion tails for the purpose of enhancing polyelectrolyte precipitation. Negatively charged aspartic acid tails and positively charged polyarginine tails were added to β-galactosidase from E. coli. These fusion tails were all shown to possess specific activity equal to the native enzyme. Gel permeation and ion exchange chromatography provided evidence as to the integrity of the tails as well as to their altered charge characteristics. All enzymes containing charged tails displayed enhanced polyelectrolyte precipitation over the native enzyme. An optimal number of charged residues, beyond which no further enhancement of precipitation was observed, was found for each type of tail to be approximately 10 residues. No interference from nucleic acids was observed in the precipitation of positively tailed β-galactosidase.
LIST OF SYMBOLS AND ABBREVIATIONS

a  peptide length constant
n  number of peptide residues
r  effective radius of polypeptide
BGCD  β-galactosidase with carboxyl aspartate fusion peptide
BGCR  β-galactosidase with carboxyl arginine fusion peptide
DEAE  diethylaminoethyl
K   coefficient
HPGPC high performance gel permeation chromatography
HPIEC high performance ion exchange chromatography
M   molecular weight
PAA poly(acrylic acid)
PAGE polyacrylamide gel electrophoresis
PEI  polyethyleneimine
SDS sodium dodecyl sulphate
V   volume
WTBG wild-type (commercial) β-galactosidase
α   separation factor
ΔM  hydrodynamic size increase

Subscripts
app  apparent
d  distribution
e  elution
INTRODUCTION

The growing demand for less expensive pharmaceuticals and other biochemical products has created a need for the development of more efficient separation processes to lower the cost of purification. Many of these products are enzymes, which often require high yields and purity levels, especially when intended for clinical use. Obtaining a pure product is particularly complicated when one considers the fact that cells consist of a complex mixture of many different components, including lipids, nucleic acids and proteins. To complicate matters further, even in such simple cells as bacteria there are on the order of 1000 different proteins (Georgiou, 1988). It has been noted by several authors that the costs of product recovery often exceed those for the fermentation itself (Bell et al., 1983; Bjurstrom, 1985; Van Brunt, 1985).

Advances in genetic engineering have launched the field of biotechnology into a new era of mass production of cheaper pharmaceutical and food products. As a result of increased expression levels and choice of microorganisms, recombinant organisms can now create mass quantities of products which were once very difficult to extract, and thus very rare and expensive (Sharma, 1986; Hoare and Dunnill, 1989). Genetic engineering has been used to enhance the separation of a protein through the fusion of a polypeptide "tail", which confers the basis for recovery, to a terminus of the protein. Examples of fusion tails which have been used to enhance separation include charged amino acids for separation by ion exchange, histidine residues for recovery by metal ion affinity, and whole proteins for recovery by ligand affinity (Hammond et al., 1991; Uhlen and Moks, 1990; Enfors et al., 1990; Ford et al., 1991; Ladish et al., 1990; Sherwood, 1990; Brewer and Sassenfeld, 1985; Parker et al., 1990). Many fusion tails have been shown not to
interfere with the activity of the enzymes and some may even protect against proteolytic degradation (Shine et al., 1980; Stanley and Luzio, 1984; Moks et al., 1987; Hammarberg et al., 1989). The majority of studies performed thus far have concentrated on enhancement of chromatographic separations, yet the technology is readily adaptable to application by precipitation.

Precipitation is one of the primary methods used to achieve fractionation during product recovery. In common practice, precipitation is used during the early stages of downstream processing to achieve partial purification of the product as well as a reduction in volume (Boing, 1982). This in turn results in lowered costs due to the subsequent processing of lower volumes. Traditionally, selectivity can be achieved by altering the choice of precipitating agent or the solvent characteristics.

Examples of polyelectrolytes commonly used as protein precipitants include the polyanions poly(acrylic acid) (PAA) and carboxymethyl cellulose (CMC), and the polycation poly(ethyleneimine) (PEI). The net effect of the electrostatic repulsion between protein molecules is minimized upon complexation between the polyelectrolyte and protein (Sternberg, 1970). Advantages of the method include high removal efficiencies and retention of enzymatic activity (Sternberg and Hershberger, 1974; Clark and Glatz, 1990). Very low amounts of polyelectrolyte (0.05-0.10% wt/vol) are required and the fractionation potential is good (Scopes, 1987). Reclamation and recycling of the polyelectrolytes can also be accomplished (Naeher and Thum, 1974; Bozzano, 1989). A disadvantage is the associated increase in susceptibility of the protein to thermal denaturation (Hidalgo and Hansen, 1971; Gekko and Noguchi, 1978).

In this work, genetically altered proteins were used to study the enhancement of protein purification using polyelectrolyte precipitation. The model
proteins used were β-galactosidase from *Escherichia coli* and glucoamylase from *Saccharomyces cerevisiae*. β-galactosidase has been altered by the addition of positively and negatively charged peptides (tails) to the carboxyl ends of the protein's four subunits. Glucoamylase has been modified by the addition of negatively charged peptides to either terminus of the enzyme. Only the results for β-galactosidase will be discussed here. The results for glucoamylase can be found elsewhere (Suominen et al., 1992).

Active β-galactosidase from *E. coli* is a tetrameric enzyme consisting of four identical subunits of 1023 amino acids each, yielding an approximate molecular weight of 470 kDa (Kalnins et al., 1983). A schematic of β-galactosidase biosynthesis is shown in Figure 1. Dissociation of subunits results in loss of protein functionality for β-galactosidase. The enzyme was originally chosen for its ability to be modified as a fusion protein without loss of activity, in addition to its simple assay (Zhao et al., 1990). β-galactosidase also has the advantage that it is resistant to proteolysis (Sassenfeld, 1990).

Positively charged fusion tails have previously been shown to enhance the recovery by ion exchange chromatography of a genetically modified small protein, β-urogastrone (Sassenfeld and Brewer, 1984; Brewer and Sassenfeld, 1985). Similarly, negatively charged aspartic acid tailed β-galactosidase fusions showed improved separation behavior relative to wild-type β-galactosidase on an ion exchange column (Zhao et al., 1990; Niederauer et al., 1991). Enhanced separation was also achieved using PEI to precipitate negatively charged aspartic acid tailed β-galactosidase fusions from crude cell extracts (Parker et al., 1990). β-galactosidase
Figure 1. A schematic of β-galactosidase biosynthesis
precipitations from these cell extracts using PEI exhibited interference from nucleic acids. PEI was found to precipitate the nucleic acids. Earlier work had shown selective removal of nucleic acids from extracts by PEI precipitation, with significant (15-20%) coprecipitation of proteins (Atkinson and Jack, 1973). The effect has been supported by charge-balance calculations which have shown the precipitation to result from the binding of negatively charged nucleic acids to positively charged proteins and PEI (Cordes, 1987).

To quantitatively characterize the PEI precipitations, the separation factor ($\alpha$) was calculated by Parker et al. (1990) as

$$\alpha = \frac{[\beta\text{-galactosidase]}_{\text{in precipitate}}}{[\text{total protein}]_{\text{in precipitate}}} \times \frac{[\beta\text{-galactosidase]}_{\text{in extract}}}{[\text{total protein}]_{\text{in extract}}}$$

A separation factor greater than unity indicates an enrichment of $\beta$-galactosidase in the precipitate. PEI treatments of crude extracts resulted in no enrichment of any of the fusion proteins in the precipitate. This is a direct result of the preferential precipitation of nucleic acids by PEI. Parker et al. (1990) found that precipitation of nuclease-treated extracts yielded no improvement for a fusion tail of 5 aspartic acid residues relative to the control enzyme, both of which had separation factors near or less than unity. A fusion tail containing 11 aspartic acid residues yielded separation factors up to 3.4. An even longer fusion tail containing 16 aspartic acid residues yielded results similar to tail with 11 residues. Extracts were also treated with nuclease followed by diafiltration to further reduce interference from nucleic acids. The two-step treatment resulted in a reduction in the amount of PEI needed to initiate precipitation, as well as an enhancement in the separation of all fusion $\beta$-
galactosidase proteins. An optimal separation factor of 1.65 was observed for the control enzyme and the tailed enzyme containing five charged residues. The fusion enzyme with 11 charged residues in the tail yielded a separation factor of 5.34.

In order to avoid the interference from nucleic acids, a series of positively charged poly(arginine) tails on β-galactosidase have been constructed. Precipitation of such positively charged fusions with negatively charged polyelectrolytes has been successful for naturally basic proteins such as lysozyme (Clark and Glatz, 1987; Shieh, 1989). The ability to exclude nucleic acids from such precipitates has not been assessed.
MATERIALS AND METHODS

Polyethyleneimine (55 kDa) in a 50 wt% solution in water was purchased from Aldrich Chemical Co. (Milwaukee, WI). BCA Protein Reagent and bovine serum albumin protein standard were purchased from Pierce Chemicals (Rockford, IL). Poly(acrylic acid) (450 kDa) was obtained from Polysciences (Warrington, PA) as a powder. All other chemicals and proteins were purchased through Sigma Chemicals (St. Louis, MO).

Construction of Enzymes

β-galactosidase manipulations were carried out on the pUR290 plasmid (Rüther and Müller-Hill, 1983) using standard DNA methodology (Sambrook et al., 1989; Zhao et al. 1990). Oligonucleotides were synthesized at the Iowa State University Nucleic Acid Facility using a Biosearch 8750 synthesizer from Applied Biosystems. The synthetic restriction fragments were designed and inserted between the BamHI and EcoRI sites at the 3' end of the lacZ coding region. The resulting carboxyl terminal oligonucleotide and corresponding protein sequences are depicted in Table 1.

Genetic modification of β-galactosidase resulting in fusions containing highly charged anionic peptides was originally carried out by Zhao et al. (1990) using the pUR290 plasmid to construct fusion proteins designated T290, T1, T2, and T3. These fusion proteins possessed carboxyl terminus anionic tails which had 1, 5, 11, and 16 aspartic acid residues, respectively. T290 has effectively zero net additional charges compared to commercial (wild-type) β-galactosidase due to the terminal arginine on the tail. For clarification, the designations have since been changed to BG290, BGCD5, BGCD11, and BGCD16, respectively. Abbreviations used for the
Table 1. The pUR290 vector (Rüther and Müller-Hill, 1983) and the sequences of the fusion tails at the carboxyl terminus of β-galactosidase (Zhao et al., 1990). The restriction sites, BamHI and HindIII, used to insert the DNA cassettes are underlined. Both the genetic and protein sequences are given. The *** indicates a stop codon which terminates protein translation.

<table>
<thead>
<tr>
<th></th>
<th>BamHI</th>
<th>HindIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUR290:</td>
<td>CAA AAA GGG GAT CCG TCG ACC TGC AGC CAA GCT TAT CGA TGA</td>
<td>Gln Lys Gly Asp Pro Ser Thr Cys Ser Gln Ala Tyr Arg ***</td>
</tr>
<tr>
<td>BGCD5:</td>
<td>CAA AAA GGG GAT CCG ATG GCA GAC GAC GAT GAC TAC TAG AAG CTT</td>
<td>Gln Lys Gly Asp Pro Met Ala Asp Asp Asp Tyr ***</td>
</tr>
<tr>
<td>BGCD11:</td>
<td>CAA AAA GGG GAT CCG ATG GCA GAC GAC GAT GAC GAT GAC GAT GAC GAT GAC TAC AAG CTT</td>
<td>Gln Lys Gly Asp Pro Met Ala Asp Asp Asp Asp Tyr ***</td>
</tr>
<tr>
<td>BGCD16:</td>
<td>CAA AAA GGG GAT CCG ATG GCA GAC GAC GAT GAC GAT GAT GAT GAT GAT GAT TAC TAG AAG CTT</td>
<td>Tyr ***</td>
</tr>
<tr>
<td>BGCR0:</td>
<td>CAA AAA GGG GAT CCG ATG CGA TAC TGA ACC TTA</td>
<td>Gln Lys Gly Asp Pro Met Ala Tyr ***</td>
</tr>
<tr>
<td>BGCD1:</td>
<td>Gln Lys Gly Asp Pro Met Ala Arg Arg Arg Arg Arg Arg Ser Tyr ***</td>
<td></td>
</tr>
<tr>
<td>BGCR5:</td>
<td>CAA AAA GGG GAT CCG ATG CGA CGT CGT CGC CGT CGT AGA TCT TAC TAG AAG CTT</td>
<td>Gln Lys Gly Asp Pro Met Ala Asp Asp Asp Asp Asp Asp Asp Tyr ***</td>
</tr>
<tr>
<td>BGCR10:</td>
<td>CAA AAA GGG GAT CCG ATG CGA CGT CGC CGT AGA CGA CGT CGC CGC CGT CGC TAC AGA AAG CTT</td>
<td>Gln Lys Gly Asp Pro Met Ala Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Tyr ***</td>
</tr>
<tr>
<td>BGCR15:</td>
<td>CAA AAA GGG GAT CCG ATG CGA CGT CGC CGT AGA CGA CGT CGC CGC CGC CGC CGC CGC CGT</td>
<td>Arg Tyr ***</td>
</tr>
<tr>
<td></td>
<td>CGC TAC AGA AAG CTT</td>
<td></td>
</tr>
</tbody>
</table>
enzymes are BG ≡ β-galactosidase, 290 ≡ base plasmid, C ≡ carboxy terminus, D ≡ aspartic acid, R ≡ arginine, and # ≡ number of residues.

**Enzyme Production**

Poly(aspartic acid) tailed β-galactosidase was produced in the *E. coli* Y1089-1 strain. Poly(arginine) tailed β-galactosidase was produced in the *E. coli* SF100 strain, generously supplied by George Georgiou. *E. coli* was grown in LB medium (Sambrook et al., 1989) supplemented with 1 mg/ml glycerol and 100 μg/ml ampicillin at 37°C. To overcome plasmid instability in SF100, the fermentation broth was periodically centrifuged at 3600 g for 10 minutes and the cell pellet resuspended in fresh media. By replacing the media, β-lactamase, which degrades ampicillin, was removed from the media so that ampicillin would not quickly be depleted in the fresh media.

**Sample Preparation**

**Crude extract**

Once the cell density in the fermentation reached an absorbance value at 600 nm of 0.8, the cells were placed on ice. The cells were centrifuged at 3600 g for 10 minutes at 4°C. The cell pellet was washed twice with 10 mM sodium acetate, pH 5.4 (pH 5.7 for purified enzyme production), before being resuspended. The cells were lysed using six 30 second bursts of sonication on ice. Centrifugation at 12000 g was used to remove the cell debris. The resulting clear solution was termed crude extract and used immediately in precipitations or further processed to recover purified enzyme.
Purified enzyme

Crude extracts were fractionated using ammonium sulfate precipitation to 50% saturation (30 g per 100 ml) followed by centrifugation for 30 minutes at 12000 g and 4°C. The pellet was resuspended in TNM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol) and dialyzed (Spectropor 12 kDa MWCO, Spectrum Medical Industries, Inc., Los Angeles, CA) against TNM at 4°C. The dialyzed sample was loaded onto a p-amino-β-D-thiogalactopyranoside agarose affinity column (Steers and Cuatrecasas, 1974). Bound protein was eluted with 1.0 M sodium borate, pH 10. Fractions containing the greatest activity were pooled and dialyzed against 20 mM sodium acetate, pH 5.7, at 4°C. Solutions were stored at 4°C prior to use.

Protein and Activity Assays

Protein content was determined using the BCA (bicinchonic acid) Protein Assay from Pierce Chemicals. The BCA protein reagent was prepared immediately prior to use. Bovine serum albumin (BSA) from Pierce Chemicals was used as the protein standard.

β-galactosidase activity was determined according to the end-point assay of Miller (Miller, 1972). The colorimetric assay is based on the hydrolysis of o-nitrophenyl β-D-galactoside by β-galactosidase to galactose and o-nitrophenol. A unit of activity is defined as one μmole of o-nitrophenol formed per minute at 30°C.

Nucleic Acid Assays

Determination of the nucleic acid content in crude extracts was made primarily by monitoring the absorbance at 280 and 260 nm. The ratio of absorbances was used to calculate the nucleic acid content relative to total protein concentration (Robyt and White, 1987; Layne, 1957). Corrections were made for the
relative ratios of nucleotides in \textit{E. coli} (Fredericq et al., 1961; Woodruff et al., 1973). The nucleic acid concentration determined in this manner was confirmed by using the orcinol assay (Herbert et al., 1971).

**SDS-PAGE**

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the procedure of Sambrook et al. (1989). Gels were made using 7.5\% acrylamide and run at a constant current of 30 mA. Coomassie blue or silver staining (Bio-Rad Silver Stain Plus) were used to stain the proteins.

**High Pressure Liquid Chromatography**

**Gel permeation**

All high pressure gel permeation chromatography (HPGPC) analyses were performed using 0.1 M phosphate buffer, pH 7.3, with sodium chloride concentrations of 0.0 and 0.3 M. Three different columns were used in the experiments. The operating conditions are given in Table 2. The eluant was monitored by absorbance at 280 nm. Fractions were collected every minute and tested for \(\beta\)-galactosidase activity. Standard proteins were run on each column to

### Table 2. Columns and operating conditions used in HPGPC analysis of \(\beta\)-galactosidase fusions.

<table>
<thead>
<tr>
<th>Column</th>
<th>Flow Rate ml/min</th>
<th>Injection Volume (\mu)l</th>
<th>Sample Concentration mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synchrom GPC300</td>
<td>0.5</td>
<td>20, 100</td>
<td>0.5-2.0</td>
</tr>
<tr>
<td>Beckman TSK5000PW</td>
<td>0.4, 1.0</td>
<td>20, 100</td>
<td>1.0</td>
</tr>
<tr>
<td>Beckman TSK3000SW</td>
<td>0.4</td>
<td>20</td>
<td>0.5-2.0</td>
</tr>
</tbody>
</table>
obtain calibration curves. Columns were obtained from Supelco, Inc. (Bellefonte, PA) and Beckman (San Ramon, CA).

Ion exchange

The column used for high performance ion exchange chromatography (HPIEC) analyses was a Supelco column (Bellefonte, PA) with Toyopearl TSK DEAE 5PW packing. This packing was chosen due to its large pore size of 1000Å. Ladisch et al. (1989) and Yamamoto et al. (1987) had previously used Toyopearl 650M packing, a preparative version of the DEAE 5PW, to achieve separation of β-galactosidase. DEAE functional groups were also used by Craven et al. (1965) to purify β-galactosidase. Previous efforts using an Aquapore AX-300 (Pierce, Rockford, IL) silica based column with a pore size of 300Å were unsuccessful. This was most likely due to the smaller pore size of the column matrix.

The flow rate was 0.5 ml/min. The buffer was 0.1 M phosphate, pH 5.7. Injections of 20 μl were made using 1 mg/ml affinity-purified enzyme solutions. A sodium chloride salt gradient from 0.0 M to 0.5 M over 30 minutes was used to invoke elution of the bound proteins. The eluant was monitored by absorbance at 280 nm. Collected fractions of 0.5 ml were tested for β-galactosidase activity.

Polyethyleneimine Assay

The PEI content of the precipitation supernatants was determined using turbidimetric titration with BSA. To 500 μl of 1.0 mg/ml BSA (in 0.1 M sodium acetate, pH 6.5), 500 μl of the supernatant was added and allowed to reach maximum turbidity (80 minutes) at room temperature. Various concentrations of PEI in the same ionic strength of the supernatant being assayed were used as standards. The turbidity was measured at 380 nm.
Polyelectrolyte Precipitation

For the precipitation of BGCR from crude cell extracts, stock polyelectrolyte solutions were made by dissolving PAA in 10 mM sodium acetate, pH 5.40, to 0.1% w/v. Crude cell extracts were diluted to 1.0 mg/ml in the same buffer. Precipitations were carried out in eppendorf tubes with 0.5 mg total protein in a final volume of 750 μl. After PAA was added to the extract, the tubes were vortexed for 20 seconds and placed on an orbital shaker at room temperature for 15 minutes. Precipitates were removed by centrifugation at 12,000 rpm for 15 minutes in a microcentrifuge. The β-galactosidase activity and protein and nucleic acid concentrations of the supernatant were determined. Pellets were washed and then dissolved by vortexing in 1.0 M, pH 6.0, sodium acetate buffer. The resulting enzyme solution was analyzed for protein and β-galactosidase activity.

Precipitations of purified poly(aspartic acid) tailed β-galactosidase were performed using PEI. Stock solutions were made by diluting PEI to 0.005% w/v in pH 5.7 sodium acetate buffer of the appropriate ionic strength. Enzyme solutions (0.5 mg/ml) were prepared in pH 5.7 sodium acetate of the appropriate ionic strength. Total volume of precipitation was 1.0 ml containing 0.25 mg protein. The remainder of the procedure was the same as was used for PAA precipitations from crude extracts.
RESULTS AND DISCUSSION

Enzyme Production

To control expression of BGCD fusion proteins, the *E. coli* strain *F'llrecA* was originally used. It contains the lacI repressor (Koenen et al., 1985), and can be induced using isopropyl β-D-thiogalactopyranoside. This strain was found to be unstable, in that it often lost the F' episome. These same plasmids are now being expressed constitutively in the *E. coli* strain *Y1089-1*. Constitutive expression avoids the induction step during growth, thus simplifying the fermentation. Yields are lower (up to 20% of total soluble protein) than those obtained using *F'llrecA* (up to 40% of total soluble protein), but there appears to be no instability with respect to the plasmid.

Expression of the BGCR fusion proteins was initially performed in the *E. coli* *Y1089-1* strain, which is deficient in the Lon protease. The absence of the Lon protease increases the stability of foreign or abnormal proteins in *E. coli* (Winnacker, 1987). Despite this, the tails were degraded by proteases inherent to the strain. Expression and faithful recovery of the fusion proteins were obtained in the *E. coli* strain *SF100*, a constitutive strain which is deficient in the OmpT protease. For the BGCR0 and BGCR5 fusion proteins, the plasmid appears stable in *SF100* and average yields are comparable to those of BGCD (up to 15% of total protein). In the production of BGCR10 and BGCR15 fusion proteins, however, the plasmid appears to be unstable and average yields were less than 1% of total protein. Supplementing the fermentation broth with arginine did not result in any improvement in yield. Yields comparable to BGCD0 and BGCD5 were obtained by frequently replacing the broth with fresh broth containing ampicillin, which permitted only those cells containing the β-galactosidase encoding plasmid to grow.
BGCR10 and BGCR15 furthermore proved extremely difficult to purify. Efforts to optimize affinity chromatography by altering buffer conditions as well as preceding the affinity step with gel permeation and ion exchange chromatography did not increase the purity of the final product. Only a very small sample of purified BGCR15 could be obtained for analysis.

**Enzyme Characterization**

Characterizations of the β-galactosidase fusions were performed to determine whether the tails were intact, whether they were located at a position on the surface of the enzyme which is accessible to other molecules, and whether activity was retained.

**Specific activity**

The specific activities of the fusion proteins were found to be nearly equal to that of wild type β-galactosidase (WTBG), except for BGCD16, which has about one-half the activity (Table 3). This indicates that the shorter tails do not interfere with the active site nor do they severely decrease enzyme stability. As for BGCD16, the anomaly of its lower activity is only one of the behavioral differences of this fusion enzyme, which will be discussed in greater detail in the following sections.

**SDS-PAGE**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to check the relative purity of affinity purified fusion proteins. A typical gel displaying the purity of BGCD5, BGCD11, and BGCD16 compared to that of WTBG is shown in Figure 2. The samples have been loaded in excess so that the impurities can be easily seen. The thickest band appearing at 116 kDa is that of the monomeric β-galactosidase subunits. Other bands which appear in the purified
Figure 2. A typical SDS-PAGE of affinity purified β-galactosidase

- Myosin, 205 kDa
- β-Galactosidase, 116 kDa
- Phosphorylase B, 97 kDa
- Albumin, Bovine, 66 kDa
- Albumin, Egg, 45 kDa
Table 3. Specific activities of carboxyl tail fusion β-galactosidase\textsuperscript{a}

<table>
<thead>
<tr>
<th>Tail</th>
<th>Units/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTBG</td>
<td>296 000</td>
</tr>
<tr>
<td>BGCD5</td>
<td>263 000</td>
</tr>
<tr>
<td>BGCD11</td>
<td>264 000</td>
</tr>
<tr>
<td>BGCD16</td>
<td>111 000</td>
</tr>
<tr>
<td>BGCR0</td>
<td>270 000</td>
</tr>
<tr>
<td>BGCR5</td>
<td>295 000</td>
</tr>
<tr>
<td>BGCR15</td>
<td>294 000</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data from duplicate assays deviated less than 3\% from each other and were averaged. Numbers given for WTBG and BGCD are those of Zhao et al. (1990) and were found to be reproducible. β-galactosidase units are those of Miller (1972).

Extract lanes have approximate molecular weights of 106, 93, 89, and 56 kDa. These bands are typical of products obtained from the affinity matrix and are indicative of non-specific binding to the matrix.

**Gel permeation HPLC**

The three different columns tested all yielded the same general trend, as seen in Table 4: increasing tail length resulted in shorter elution times, $V_e$, corresponding to an increased hydrodynamic size. The calibration plot for the TSK SW 3000 column is shown in Figure 3. The distribution coefficient, $K_d$, describes the elution time of the sample as a fraction of the period between the elution of particles at the minimum and maximum size limits of the column. BGCR10 and BGCR15 were not analyzed since affinity purified samples were not available.
Figure 3. Calibration plot for the TSK SW 3000 column for globular proteins: ▼, BGCR5; ■, BGCD1; ▲, BGCD5; ●, BGCD11; ◆, BGCD16; X, protein standards (protein standard near β-galactosidase which is not on calibration line is apoferretin)
Table 4. Results of HPGPC for β-galactosidase fusions. Results shown were obtained in buffer containing 0.3M NaCl.

<table>
<thead>
<tr>
<th></th>
<th>BGCR5</th>
<th>BGCD1</th>
<th>BGCD5</th>
<th>BGCD11</th>
<th>BGCD16</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPC300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ve</td>
<td>--</td>
<td>4.35</td>
<td>4.20</td>
<td>4.10</td>
<td>4.15</td>
</tr>
<tr>
<td>5000PW</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ve</td>
<td>--</td>
<td>7.71</td>
<td>7.69</td>
<td>7.66</td>
<td>7.62</td>
</tr>
<tr>
<td>3000SW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ve</td>
<td>14.35</td>
<td>14.47</td>
<td>14.00</td>
<td>13.76</td>
<td>13.62</td>
</tr>
<tr>
<td>Kd</td>
<td>0.133</td>
<td>0.140</td>
<td>0.112</td>
<td>0.0978</td>
<td>0.0902</td>
</tr>
<tr>
<td>M_{add}</td>
<td>494000</td>
<td>468000</td>
<td>576000</td>
<td>642000</td>
<td>680000</td>
</tr>
<tr>
<td>M</td>
<td>471900</td>
<td>468400</td>
<td>470500</td>
<td>473700</td>
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<tr>
<td>%ΔM</td>
<td>5.55</td>
<td>0.00</td>
<td>23.1</td>
<td>37.2</td>
<td>45.3</td>
</tr>
</tbody>
</table>

With respect to the estimates of the apparent molecular weight, M_{app}, based on K_{d} values, only the values from the Beckman Spherogel TSK 3000 SW column were calculated. This column provided the best resolution between the tails. Even though the TSK 5000 PW column can achieve separation over a wider range of molecular weights, it has been shown that the TSK 3000 SW column yields better separations over its separation range (Kato, 1984). β-galactosidase eluted at the higher resolution limit on the GPC 300 column. Notable is the fact that whereas the largest real increase in molecular weight was approximately 1.5% for BGCD16, estimates from K_{d} values yield a corresponding hydrodynamic size increase, ΔM, of 45.3%. Estimates of the of the hydrodynamic size of the proteins were made based on the apparent molecular weights, M_{app}, using the correlation of Teller (1976). The difference between estimated radii of tailed and untailed β-galactosidase was taken as an estimate of the root mean square end-to-end length of the tail. These estimates were 6.0 Å for BGCD5, 9.4 Å for BGCD11, and 11.2 Å for BGCD16. This length
should follow the formula $r = a\sqrt{n}$ for random conformation polymer chains (Rodriguez, 1982), where $r$ is the effective radius of the polypeptide, $a$ is the peptide length constant, and $n$ is the number of peptide residues. Using this formula, an average value for $a$ was found to be 2.95 Å, giving lengths of 5.9 Å, 9.3 Å, and 11.4 Å, respectively. This compares well with an $a$ value of 3.74 Å for fully extended peptides. It can therefore be seen that the observed size exclusion behavior is consistent with the hypothesis that the tails extend into the surrounding solution.

**Ion exchange HPLC**

High performance ion exchange chromatography (HPIEC) was conducted to confirm the results obtained from HPGPC, as well as to provide evidence as to the integrity of the fusion peptide tails. A comparison of the chromatograms obtained is shown in Figure 4. One can see from the chromatograms that for all the tails studied, except BGCD16, the trend of elution is as expected from the additional fusion peptide charges. BGCD16 elutes at a much lower ionic strength than would be predicted from its net estimated tetramer charge. The elution pattern of BGCD16 is also notably different in that it displays a high degree of bandspreading. In general, however, the more negatively charged proteins bind more strongly to the column and thus require higher salt concentrations for elution.

Attempts to analyze BGCR10 and BGCR15 on the DEAE anion exchange column failed. These fusion proteins had been expected to bind with the column, according to the binding behavior of the other tails, yet neither enzyme revealed any elution peaks, as detected by absorption at 280 nm. Both were, however, detected by assaying the collected fractions for activity. Both enzymes began eluting at ionic strengths between those required for elution of BGCR5 and BGCR0, and continued eluting up to the final 0.5 M salt concentration. A possible
Figure 4. High performance ion exchange chromatography analysis of β-galactosidase
explanation may be complexation between the longer arginine fusion peptides and the divalent phosphate anions present in the buffer. Ichimura et al. (1978) found that poly(L-arginine) associates with divalent anions such as phosphate to form helical structures. This would effectively neutralize the charge of the poly(arginine) tail, reducing or eliminating electrostatic interactions of the tail with the ion exchange matrix. The results did not, however, improve upon switching the buffer to sodium acetate, a monovalent buffer. Further attempts at the characterization of BGCR10 and BGCR15 using a MEMSEP CM 1000 cation exchange cartridge (Millipore) under a variety of buffer conditions were also not successful.

**Crude Extract Precipitation**

Control of the pH was found to be critical in precipitations of BGCR crude extracts with PAA. No precipitation was observed at pH values exceeding 5.6. Estimations of enzyme charge from the Henderson-Hasselbalch equation using amino acid pK values from Stryer (1988) showed that BGCR0 did not obtain a net positive charge until the pH was dropped below 5.5. Reproducible results were obtained at pH 5.40±0.02. Tight pH control was necessary since at pH values below 6, wild-type β-galactosidase becomes progressively insoluble. At pH 5.40, isoelectric precipitation of protein was less than 6% and consisted primarily of β-galactosidase. The high degree of pH control was required since upon the addition of even small amounts of PAA, deviations in the amount precipitated could exceed 20% for pH deviations as small as 0.1.

Peptide tails consisting of 5 and 10 arginine residues resulted in increased precipitation with increasing tail length, yet no further enhancement was achieved with 15 residues (Figure 5). BGCR comprised only 1% of the total protein in the crude extract, so total protein in Figure 5 is indicative of other proteins; only small
Figure 5. PAA precipitation of BGCR crude extracts at pH 5.4: Open symbols, total protein; closed symbols, activity; ■, □, BGCR0; △, Δ, BGCR5; ●, ○, BGCR10; ◆, ◆, BGCR15
amounts of other proteins were precipitated by PAA. The total protein precipitated increased up to a dosage of 10 to 20 mg/g, and then decreased at higher dosages. This resolubilization of protein resulted in large purification factors, as shown in Figure 6. However, no activity could be recovered from the precipitants. This may be because the precipitation must be carried out very close to the isoelectric point, where β-galactosidase irreversibly denatures, in order to overcome the high net negative charge of β-galactosidase.

Interference from nucleic acids, as was evident with BGCD fusions, was not observed in this system. Some nucleic acid precipitation is observed, yet it is thought that this is due to non-specific associations with proteins. The nucleic acid precipitation behavior parallels that of the total protein, not that of β-galactosidase. Figure 7 shows the trend of nucleic acid precipitation determined by the absorbance ratio at 280/260 nm as well as by the orcinol assay: the results for the two assays were essentially identical.

Purified Enzyme Precipitation

Precipitations of purified BGCD fusion proteins with PEI were conducted at various ionic strengths. BGCD16 was not used in purified enzyme precipitation studies since it displayed abnormal behavior in the characterization studies. The expected enhancement due to tail length was observed to be similar to previous results (Parker et al., 1990; Zhou et al., 1990): greater tail length resulted in increased precipitation. Precipitations of wild-type and CD1 β-galactosidase yielded the expected behavior in which increasing the ionic strength results in a decrease in precipitation (Figures 8 and 9). For BGCD5 and BGCD11, however, the opposite trend was observed for ionic strengths of 20, 100, and 200 mM (Figures 10
Figure 6. Separation factors from PAA precipitations of BGCR crude extracts: ■, BGCR0; ▲, BGCR5; •, BGCR10; ◆, BGCR15
Figure 7. Nucleic acid precipitation from PAA precipitations of BGCR crude extracts: ■, BGCR0; ▲, BGCR5; ●, BGCR10; ◆, BGCR15
Figure 8. PEI precipitation of commercial β-galactosidase in sodium acetate, pH 5.7: ■, 20 mM; ○, 100 mM; ◆, 200 mM
Figure 9. PEI precipitation of purified BGCD1 in sodium acetate, pH 5.7: ■, 20 mM; ○, 100 mM; ◇, 200 mM.
Figure 10. PEI precipitation of purified BGCD5 in sodium acetate, pH 5.7: ■, 20 mM; ●, 100 mM; ◇, 200 mM
and 11). The yield of precipitation increased with increasing ionic strength for these two fusion proteins.

It is proposed that the increase in precipitation upon increasing ionic strength is due to the presence of the tails, which represent a region of high charge density which is readily accessible. Assuming that all of the polyelectrolyte is contained in the precipitate (Shieh, 1989), charge balance calculations show the residual charge of the complex to be high. A high complex residual charge was found in the precipitation of lysozyme by CMC (Clark and Glatz, 1992). As the ionic strength is increased, the solubility of the complex is decreased through ionic shielding which reduces intermolecular repulsion. This would normally also be offset by an accompanying decrease in binding strength between the polyelectrolyte and enzyme which would solubilize the enzymes. For enzymes containing charged polypeptide fusions, however, it has been shown by ion exchange chromatography that the presence of the tails also results in stronger binding (Sassenfeld and Brewer, 1984; Brewer and Sassenfeld, 1985; Niederauer et al., 1991; Zhao et al., 1990).

Higher ionic strengths were required to elute the tailed enzymes from ion exchange columns. An increase in precipitation upon increasing the ionic strength can also be found in the precipitation of two oppositely charged polyelectrolytes (Tsuchida et al., 1975) or the flocculation of silica with PEI (Lindquist and Stratton, 1976). In both cases, the increase in precipitation upon increasing ionic strength was attributed to the resulting increase in ionic shielding which decreased the electrostatic repulsion between the complexes. Tsuchida et al. (1975) also cited the increased hydrophobic character of the polyelectrolyte complexes as being a cause of aggregation.

Recovery of activity from the precipitates was found to be complete in all cases (data not shown). However, in the case of CD5 and CD11, the ionic strength
Figure 11. PEI precipitation of purified BGCD11 in sodium acetate, pH 5.7: ■, 20 mM; ○, 100 mM; ◇, 200 mM
of the redissolution buffer (1.0M) was insufficient. Dissolution of the precipitate was accomplished with 2.5M Tris-HCl buffer, pH 7.0. This further indicates that the strength of binding due to the presence of the high charge density of the tails is increased over that of the native enzyme.

No PEI could be detected in any of the supernatants of the precipitation experiments (results not shown). This indicates that all of the PEI is present in the precipitate and that even at dosages greater than that required for complete precipitation, all of the PEI is incorporated into the protein-polyelectrolyte complex. Similar results have been reported for protein precipitations with PAA (Shieh, 1989) and CMC (Hill and Zadow, 1974 and 1978).

That the fusion tailed enzymes actually displayed greater differences in precipitation compared to BGCD1 at higher ionic strengths, as seen in Figure 12, suggests that the purification factors obtained in experiments using crude extracts at 70 mM ionic strength by Parker et al. (1990) could be improved by increasing the ionic strength. Increasing the ionic strength, however, would still not circumvent the interference from nucleic acids, as these have been shown to precipitate even at 0.5 M (Jendrisak, 1987; Atkinson and Jack, 1973).
Figure 12. Comparison of PEI precipitation curves of purified BGCD in 200 mM sodium acetate, pH 5.7:
■, BGCD1; ▲, BGCD5; ●, BGCD11
CONCLUSIONS

Enhancement of the precipitation of proteins with polyelectrolytes through the genetic fusion of charged polypeptides has been demonstrated using \( \beta \)-galactosidase as a model protein. The degree of enhancement was found to increase with the length of the polypeptide tail up to an optimum length, beyond which no further enhancement was observed. For either positively or negatively charged fusion tails, the optimal tail length was approximately 10 residues.

All fusion proteins were found to have activity equivalent to the wild-type enzyme, with the exception of the poly(aspartic acid) fusion containing 16 charged residues. Characterization of the fusion proteins by gel permeation and ion exchange liquid chromatography demonstrated that the polypeptide tails, with the possible exception of the poly(aspartic acid) fusion containing 16 charged residues, were intact and accessible to the surrounding media.

Interference from nucleic acids in crude extract precipitations evidenced with poly(aspartic acid) fusions was overcome by using oppositely charged poly(arginine) fusions. However, precipitation of poly(arginine) \( \beta \)-galactosidase fusions with poly(acrylic acid) had to be carried out under tight pH control near the isoelectric point of \( \beta \)-galactosidase. Furthermore, no activity could be recovered from the precipitates. The inactivation was most likely due to the low operating pH.

Conversely, full activity was recovered from poly(ethyleneimine) precipitations of purified poly(aspartic acid) \( \beta \)-galactosidase fusions at pH 5.7. No decrease in precipitation was observed with these fusion proteins upon increasing the ionic strength, as was observed for the control and wild-type enzymes. It is proposed that the high charge density of the tails results in strong binding to the
polyelectrolyte. These polyelectrolyte-tail bonds are not disrupted at the ionic strengths investigated in this study. As a result of their resistance to increases in ionic strength, these fusion proteins would exhibit even better separation at higher ionic strengths.
ACKNOWLEDGMENTS

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REFERENCES


PAPER III: MODEL OF THE POLYELECTROLYTE PRECIPITATION OF GENETICALLY ENGINEERED ENZYMES POSSESSING CHARGED POLYPEPTIDE TAILS
MODEL OF THE POLYELECTROLYTE PRECIPITATION OF
GENETICALLY ENGINEERED ENZYMES POSSESSING
CHARGED POLYPEPTIDE TAILS

by

Mark Q. Niederauer and Charles E. Glatz

Department of Chemical Engineering

Iowa State University
Ames, Iowa 50011

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### LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>a</td>
<td>distance of closest approach, Å</td>
</tr>
<tr>
<td>A</td>
<td>parameter in Debye-Huckel equation</td>
</tr>
<tr>
<td>B</td>
<td>constant in Debye-Huckel equation</td>
</tr>
<tr>
<td>BGCD</td>
<td>β-galactosidase with carboxy-terminal poly(aspartic acid) fusion</td>
</tr>
<tr>
<td>C</td>
<td>parameter in Debye-Huckel equation</td>
</tr>
<tr>
<td>CMC</td>
<td>carboxymethyl cellulose</td>
</tr>
<tr>
<td>D</td>
<td>dosage, g polyelectrolyte (g protein)^{-1}</td>
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<tr>
<td>f</td>
<td>functionality</td>
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<tr>
<td>F</td>
<td>parameter in Halicioglu and Sinanoglu's expression</td>
</tr>
<tr>
<td>g</td>
<td>functionality</td>
</tr>
<tr>
<td>G</td>
<td>Gibb's free energy, kcal mol^{-1}</td>
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<tr>
<td>GACD</td>
<td>glucoamylase with carboxy-terminal poly(aspartic acid) fusion</td>
</tr>
<tr>
<td>H</td>
<td>molecular surface area of the solute, Å^2</td>
</tr>
<tr>
<td>I</td>
<td>ionic strength, mol L^{-1}</td>
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<tr>
<td>K</td>
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<tr>
<td>m</td>
<td>slope</td>
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<tr>
<td>M</td>
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<tr>
<td>p</td>
<td>fraction reacted</td>
</tr>
<tr>
<td>P</td>
<td>unmodified (control) protein</td>
</tr>
<tr>
<td>PAA</td>
<td>poly(acrylic acid)</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>r</td>
<td>stoichiometric ratio of reactive groups</td>
</tr>
</tbody>
</table>
R  ideal gas constant
R_i radius of gyration of species i, Å
T  temperature, °K
V  molar volume of solvent, Å³
X  polyelectrolyte
Z  net charge
κ\*e dimensional correction for the macroscopic surface tension
Λ  intrinsic salting-in constant
ν  average binding number
σ  surface tension increment, dyn cm⁻¹
Φ  cooperativity function
ω  surface tension of pure water, dyn cm⁻¹
Ω  intrinsic salting-out constant

Superscripts
*  standard state
‘  uncharged

Subscripts
abs absolute
f  multifunctional molecule
i species i
int intrinsic
o  intercept
p  protein
t  tail peptide
x  polyelectrolyte
ABSTRACT

A model is presented for the polyelectrolyte precipitation of proteins possessing charged fusion tails. The model is based on multiple equilibria binding and accounts separately for the binding of the fusion polypeptide. The predictions of the model are compared to experimental results obtained with monomeric and multimeric fusion proteins. The enzymes investigated were various fusions of glucoamylase from *Aspergillus niger* and β-galactosidase from *Escherichia coli*, respectively. Electrostatic cooperativity is not evidenced for the binding of these negatively charged proteins to positively charged, highly branched polyethyleneimine. Qualitative agreement is achieved between the model and experimental results for the behavior of the association constants of the protein and fusion polypeptide with respect to the number of polypeptide charges, ionic strength, and polymer dosage. For the precipitation of multimeric proteins, it is proposed that the each of the fusion polypeptides acts as a strong electrostatic interaction site which can preferentially bind the enzyme to multiple polyelectrolytes, resulting in a tightly bound, crosslinked matrix. Increasing the ionic strength leads to a reduction in the electrostatic repulsion within the protein-polyelectrolyte complex. The combination of reduced electrostatic repulsion and the strong binding of the tails results in enhancement of the precipitation as the ionic strength is increased.
INTRODUCTION

The accurate modeling of separation methods offers two advantages: gaining an understanding of the mechanisms and their relative importance in effecting the separation, and accurate prediction of the conditions which will result in a desired separation. Many models of various separation processes exist. The accuracy of these models varies depending on the complexity of the process they are attempting to describe. Each of the various general methods of precipitation have been modeled to some degree [1,2]. Authors who have modeled the various aspects of polyelectrolyte precipitation of proteins include Clark and Glatz [3] and Fisher and Glatz [4]. Due to the complexity and vast number proteins, not to mention other cellular components, these models focus on defined systems.

Our work has focused on the enhancement of polyelectrolyte precipitation through the genetic fusion of charged polypeptides [5-8]. A model to account for the enhancement of precipitation as a result of these polypeptide tails is developed here. Development of the model closely follows that of Clark and Glatz [3]. Before proceeding with the deliberation of the model itself, the effects which the model is intended to account for will be presented. In their work involving the precipitation of the egg white proteins lysozyme and ovalbumin by carboxymethyl cellulose (CMC), Clark and Glatz [9] came to several conclusions concerning the effects of pH, polymer dosage, and ionic strength on protein recovery and fractionation:

1) Only proteins possessing a charge opposite to that of the polyelectrolyte are precipitated, and those of higher charge density are precipitated preferentially.
2) The efficiency of precipitation increases with protein charge; less polyelectrolyte is needed to achieve precipitation of proteins possessing higher charge.

3) Up to an optimal polymer dosage, protein removal increases with polyelectrolyte dosage. At dosages higher than the optimum, protein removal decreases. Highly charged proteins are less susceptible to the latter effect.

4) An increase in ionic strength increases the required polymer dosage to effect the same protein removal, reduces the maximum possible precipitation, and reduces the effect which precipitation pH has on protein recovery.

5) Fractional precipitation can be attained by the proper adjustment of pH or polymer dosage. If the target protein is highly charged, the efficiency of fractionation can be improved by increasing the ionic strength; the target protein will be purified to a higher degree.

An initial attempt at modeling the precipitation process as a soluble analog to ion exchange was made by Clark [10]. The model was based on Carlson's [11] model for protein ion exchange. The model incorporates phase equilibria criteria and assumes the polyelectrolyte to be entirely in the solid phase. The latter assumption has been found by Shieh [12] to be true for poly(acrylic acid) (PAA) and CMC precipitations of egg white proteins and pure lysozyme over nearly the entire dosage range. Slight deviations were observed at high and low dosages. Hill and Zadow [13,14] found the assumption to be not entirely accurate for precipitations with CMC. Clark's model was found to have several failings, even though qualitative prediction was obtained up to the optimum polyelectrolyte dosage:
1) The model predicted a significantly lower protein recovery than was found experimentally.  
2) The predicted effect of ionic strength on the protein removal was found to be much greater than experimental results.  
3) The increased solubility of protein-polymer complexes at polyelectrolyte dosages greater than the optimum was not predicted by the model.

The model which was finally proposed by Clark and Glatz [3] assumes multi-equilibrium and cooperative binding. The phenomenon where a substrate binds a variable number of ligands is defined as multi-equilibrium. Cooperative binding accounts for the effect which ligands already bound to a substrate have on subsequent binding. Whether further binding is enhanced or deterred is termed positive or negative cooperativity, respectively [3, 15, 16]. Cooperativity is an effect often observed in biological systems. A modification of the Debye-Huckel theory was used to account for the electrostatic effects responsible for cooperative binding.
DEVELOPMENT OF THE MODEL

To account for the binding of multiple ligands to a macromolecule, the theory of multiple equilibria is used. Multiple equilibria defines the case in which multiple ligand molecules can bind to each macromolecule. The model is based on the fact that the macromolecules will bind various amounts of ligand. For the purposes of modeling polyelectrolyte precipitation, the polyelectrolyte, X, will be defined as the macromolecule which has n binding sites for a protein ligand, P. The polyelectrolyte can exist in n+1 forms if interactions other than polyelectrolyte-protein interactions are neglected. Since the macromolecule can exist in many states, an average number of ligands which are bound to the macromolecule is often used to express the multiple equilibrium binding. The average binding number, v, is described by:

$$v = \frac{[P]_{\text{Bound}}}{[X]_{\text{Total}}} \quad (1)$$

where the square brackets denote molar concentrations. The binding would thus be described by n types of reactions and their respective association constants:

$$X + P \leftrightarrow XP \quad K_1 = \frac{[XP]}{[X][P]}$$

$$X + 2P \leftrightarrow XP_2 \quad K_2 = \frac{[XP_2]}{[X][P]^2} \quad (2)$$

$$\vdots$$

$$X + nP \leftrightarrow XP_n \quad K_n = \frac{[XP_n]}{[X][P]^n}$$

It follows from Equation 1 that the expression which describes this type of binding is given by
which, upon comparison with Equation 2 yields:

\[ V = \frac{\sum_{i=1}^{n} i[XP_i]}{\sum_{i=0}^{n} [XP_i]} \]  

which, upon comparison with Equation 2 yields:

\[ V = \frac{\sum_{i=1}^{n} iK_i[P]^i}{\sum_{i=0}^{n} K_i[P]^i} \]

Tanford [17] and Van Holde [15] have shown that for identical and independent binding sites, multiple equilibria binding can be described by one average association constant, reducing Equation 4 to

\[ V = \frac{nK[P]}{1 + K[P]} \]

where \( K \) is the association constant for the binding of a protein molecule to an unoccupied site on a protein-polyelectrolyte complex and \([P]\) is the molar concentration of unbound protein. This equation assumes that each of the binding sites on the macromolecule possesses the same affinity for the ligand as any other, i.e. the binding is non-cooperative. Solving for the association constant yields

\[ K = \frac{V}{[P](n - V)} \]

which corresponds to a Gibb's free energy change of

\[ \Delta G = \Delta G^0 + RT\ln K \]
Extension to Ligands Possessing Multiple Intrinsic Affinities

The genetically engineered enzymes which we are investigating possess a high charge density polypeptide tail on the surface of the enzyme, which might be expected to have a different affinity towards the macromolecule than the protein surface in general. A schematic of the precipitation of such fusion enzymes by polyelectrolytes is depicted in Figure 1. An equation for the binding between a large molecule and two ligands (small molecules or ions, in the original development), each of which possess different affinities for the large molecule, was developed by Tanford [17] and Van Holde [15]. The general expression which describes such binding is given by

\[
v = \frac{n_p K_p[P]}{1 + K_p[P]} + \frac{n_t K_t[P]}{1 + K_t[P]}
\]  

(8)

where the subscripts p and t denote the two different ligands. As used here, a single protein ligand possesses two different affinities for the polyelectrolyte, depending on whether binding is dominated by the protein itself (subscript p) or by the charged fusion tail (subscript t).

In the absence of the tail, \(n_t=0\) and Equation 8 reduces to Equation 6. The latter part of Equation 8 represents the additional protein bound to the polyelectrolyte through interactions with the tail. It then follows from Equation 8 that the determination of \(K_t\) is made relative to the control enzyme which lacks the fusion tail. \(K_t/K_p\) would thus be an indication of the strength of binding of the tail relative to that of the control protein. The increased binding strength resulting from the presence of the charged fusion peptides has already been demonstrated using ion exchange chromatography [8]. Higher ionic strengths were required to elute the tailed enzymes from the ion exchange column.
Figure 1. Schematic of polyelectrolyte precipitation for enzymes possessing charged fusion polypeptides.
The first term on the right hand side of Equation 8 describes the interaction of the unmodified protein with the polyelectrolyte. It is assumed that there is no interaction between the protein and the tail, hence the values for \( n_p \) and \( K_p \) would be the same in the presence or absence of the tail. The calculation of \( n_p \) based on charge equivalence through the assumption of a neutral complex, as was made by Clark and Glatz [3] in their model, is not made here because the size of the enzymes will sterically limit binding. The effect of steric hindrance can be demonstrated for the two enzymes studied. The hydrodynamic radii of the 470 kDa \( \beta \)-galactosidase and the 65 kDa glucoamylase were estimated to be 84.1 Å and 37.8 Å, respectively, using the correlation of Teller [18] for monomeric and multimeric proteins. The highly branched form of polyethyleneimine (PEI) used in the precipitations has been shown to assume a spherically symmetric compact shape in solution [19]. The hydrodynamic radius of the 55 kDa polyelectrolyte was estimated to be 130 Å at \( I = 0.1 \) M, using the correlation between molecular weight and size from Lindquist and Stratton [20] and Hostetler and Swanson [19]. The maximum number of proteins which could sterically interact with the polyelectrolyte was estimated by treating both the protein and polyelectrolyte as hard spheres to determine how many proteins could pack on the surface of the polyelectrolyte. This number was approximated as the number of proteins which could pack (square-pitch) on a flat surface whose area was equivalent to that of a sphere with a radius equal to the combined radii of the protein and polyelectrolyte. Using the hydrodynamic sizes given above, \( n_p \) was found to be sterically limited to 20 for \( \beta \)-galactosidase and 61 for glucoamylase. These estimates are well below the \( n_p = 28 \) and 300, respectively, found by assuming charge equivalence of the protein and PEI at the experimental pH (see Table 1).
Table 1: Estimated net charge for β-galactosidase, glucoamylase, and PEI. Net protein charges were estimated using the Henderson-Hasselbalch equation and amino acid pK values from Stryer [21]. The charge on PEI was estimated from the titration data of Kokufuta [22].

<table>
<thead>
<tr>
<th>pH</th>
<th>Molecule</th>
<th>Estimated Net Charge</th>
</tr>
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<tbody>
<tr>
<td>5.7</td>
<td>BGCD1</td>
<td>-22.5</td>
</tr>
<tr>
<td></td>
<td>BGCD5</td>
<td>-37.7</td>
</tr>
<tr>
<td></td>
<td>BGCD11</td>
<td>-60.6</td>
</tr>
<tr>
<td></td>
<td>PEI</td>
<td>625</td>
</tr>
<tr>
<td>4.5</td>
<td>GACD0</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td>GACD5</td>
<td>-5.3</td>
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<tr>
<td></td>
<td>GACD10</td>
<td>-8.1</td>
</tr>
<tr>
<td></td>
<td>PEI</td>
<td>750</td>
</tr>
</tbody>
</table>

Cooperativity as a Result of Electrostatic Interactions

If the binding of a ligand to one site does influence the affinity of other sites, the binding is said to be cooperative. Tanford [17] proposed that $K$ be defined in terms of an intrinsic association constant, $K_{int}$, and a cooperativity function, $\Phi$. The advantages of this definition are that it accounts for the effects of cooperative binding through a single function, and that the equilibrium behavior can be described in terms of a single association constant, rather than $n$ association constants. The form of the cooperativity function will depend on the nature of the interaction. For negative cooperativity, the strength of binding, $K$, will decrease as the number of bound sites, $v$, increases.
Tanford developed a model for the case where cooperativity was the result of electrostatic binding between charged species where all electrostatic interactions vanish when the net average charge of the complex, $Z$, is zero. Adapted to this situation [3], the polyelectrolyte is assumed to have $n$ identical binding sites for the protein, each of which displays cooperative binding. For this system it is convenient to define a cooperativity function with an intrinsic association constant, $K_{\text{int}}$, as

$$K = K_{\text{int}} e^{-\Phi(Z)}$$

(9)

where $K_{\text{int}}$ is the limiting value of $K$ when all of the binding sites are filled ($v=n$, $Z=0$). $K_{\text{int}}$ will now have a corresponding intrinsic standard free energy change, $\Delta G_{\text{int}}^*$ such that

$$\Delta G^* = \Delta G_{\text{int}}^* + RT\Phi(Z)$$

(10)

As defined, $\Phi(Z)$ is a positive function of $Z$ if the macromolecule and ligand possess like charge, and a negative function of $Z$ if the macromolecule and ligand possess opposite charge.

**Determination of $-\Phi(Z)$ and $K_{\text{int}}$**

To evaluate the functionality of $\Phi(Z)$, Tanford [17] proposed that it is directly related to the change in electrostatic free energy of the complex. Clark and Glatz [3] used a modification of the Debye-Huckel theory by Melander and Horvath [23], which incorporates Kirkwood's expression [24] to accommodate for high ionic strength effects, to yield for the cooperativity of binding:

$$\Phi(Z) = ZZ_p \left( A - \frac{B\sqrt{I}}{1 + C\sqrt{I}} \right)$$

(11)

The constants were evaluated for aqueous solutions at 25°C to be
where \( I \) is in mol/I, and \( R_i \) and \( a \) are in Å. The distance of closest approach, \( a \), is determined from the center-to-center distance of the macromolecule and the ligand.

The expression for \( K_{int} \) incorporated Halicioglu and Sinanoglu's [25] expression for the free energy change of cavity formation upon transferring a solute molecule into solution [3]:

\[
RT \ln K_{int} = F - \Delta G^\circ + \frac{Z_p^2}{2} \left( A - \frac{B\sqrt{I}}{1 + C\sqrt{I}} \right) + (\Omega - \Lambda)IRT.
\]

where

\[
F = [NH + 4.8N^{1/3}(\kappa^e - 1)V^{2/3}]\omega, \quad \text{and}
\]

\[
\Omega = [NH + 4.8N^{1/3}(\kappa^e - 1)V^{2/3}]\sigma/RT.
\]

The latter term on the right hand side of the equation contains the terms \( \Lambda \) and \( \Omega \), which are commonly referred to as the intrinsic salting-in and salting-out coefficients, respectively. Equation 15 is valid for either \( K_p \) or \( K_t \).

**Crosslink Formation**

The above treatment describes a situation where precipitation results from the increasing hydrophobicity of the protein/polyelectrolyte complex. For a multimeric fusion enzyme such as \( \beta \)-galactosidase an alternative solubility criteria should be considered. The versions of the enzyme possessing fusion tails would contain multiple strong electrostatic interaction sites. These sites would be capable
of forming crosslinks between enzyme-polyelectrolyte complexes by the binding of a single enzyme to multiple polyelectrolytes. The result would be a tightly bound matrix of enzyme and polyelectrolyte, analogous to the picture of affinity matrix formation in the affinity precipitation of multimeric enzymes by bis-ligands [26,27]. A schematic of this process is shown in Figure 2. In this scenario, increasing amounts of polyelectrolyte lead to larger complex sizes of decreasing solubility. The formation of an insoluble matrix would then be expected to be dependent on the amount of polyelectrolyte relative to the protein in solution, which is termed the dosage. Furthermore, these complexes would be expected to have a relatively high net charge as a result of steric limitations to binding discussed earlier. One would therefore expect the complexes to be soluble until they reach a high molecular weight through matrix formation. The binding of the polyelectrolyte to the protein would still be governed by the same equations as developed in the previous sections, yet the criteria for precipitation could now be viewed as the formation of a gel matrix of very large molecular weight.

The polyelectrolyte dosage required for gel formation/precipitation can be estimated from the theory of gel formation in polymerization reactions. In such a reaction, successively higher conversion of two types of multifunctional monomers to polymer, in which at least one monomer possesses a functionality greater than two, increases the probability of forming a network. In the network, all of the monomers of higher functionality in the reaction mass are interconnected. The point at which the statistical probability for the formation of such a network becomes one is termed the gel point. The conversion at this point has been derived for the reaction in which one type of molecule is bifunctional and the other has a
Figure 2. Precipitation resulting in matrix formation for the polyelectrolyte complexation of multimeric enzymes possessing multiple charged fusion polypeptides.
functionality of \( f > 2 \) [28-30]. For such a reaction, the fraction of higher functionality groups that have reacted at the gel point, \( p_f \), is given by

\[
\left( \frac{1}{p_f} \right)^2 = r(f - 1)
\]  

where \( r \) is the molar stoichiometric ratio of the two types of reacting groups. Precipitation would be expected to occur at a point equal to or less than the gel point as a result of the high molecular weight of the complexes formed. Experimentally, precipitation was found by Bobalek et al. [31] to occur prior to the formation of a gel in polymerization reactions.

The polymerization scenario is very similar to the formation of polyelectrolyte-protein complexes in that the polyelectrolyte and multimeric protein both act as polyfunctional monomers in a polymerization reaction. For the purpose of modeling the formation of an interconnected matrix, or gel, in polyelectrolyte precipitation, Equation 18 could be used as an approximation of the crosslinking occurring between a dimeric protein and a polyelectrolyte. If all four tails of the tetrameric \( \beta \)-galactosidase could take part in network formation, lower values for \( p_f \) would result. Equation 18 can be extended to account for this higher functionality, \( g \), of the second "monomer":

\[
\left( \frac{1}{p_f} \right)^2 = r(f - 1)(g - 1)
\]  

g > 2. It can be seen that for \( g = 2 \), Equation 19 reduces to Equation 18. The gel point can thus be calculated from Equation 19 by allowing \( f \) to be the maximum number of enzymes which can bind to a polyelectrolyte (\( f = n \)) and inserting the value of \( r \), which is a function of the dosage, \( D \):
Model Predictions

Equation 9 predicts that $\ln K_p$ should vary linearly with $Z$, having a slope of $-\Theta(Z)/Z$ and an intercept of $\ln K_{int,p}$. From Equation 11, the slope $-\Phi(Z)/Z$ should have a positive value for the interaction of a negatively charged protein or polypeptide tail with a positively charged polyelectrolyte. The absolute value of the slope would be expected to decrease as ionic strength increases, reaching a constant value at high ionic strength. Since $K_t$ is a modification of $K_p$ to account for the presence of the tail it should follow the same behavior as $K_p$. In the case of multiple-tailed enzymes, precipitation (and if binding was equated with precipitation) would furthermore be expected to be dependent on the polyelectrolyte dosage.

\begin{align*}
  r &= D \left( \frac{M_p}{M_x} \right) \left( \frac{f_x}{f_p} \right) = D \left( \frac{M_p}{M_x} \right) \left( \frac{f}{g} \right) \\
  Pf &= \left[ D \left( \frac{M_p}{M_x} \right) \left( \frac{f}{g} \right) (f-1)(g-1) \right]^{-1/2}
\end{align*}
MATERIALS AND METHODS

Two enzymes, glucoamylase and β-galactosidase, were genetically modified and used in the precipitation studies of purified enzymes. Purified enzyme solutions were used to avoid interference from other proteins. The tails of all enzymes were constructed primarily of poly(aspartic acid) and located at the carboxyl terminus of the enzyme. All precipitations were carried out in sodium acetate buffer of various ionic strengths. PEI of 55 kDa was used as the precipitant. Details as to the construction and production of the enzymes can be found elsewhere [5, 7, 8].

Glucoamylase from *Aspergillus niger* was used as the monomeric enzyme for the precipitation studies. Three different fusion enzymes were constructed from a shortened version of the enzyme. All versions were found to retain full activity. The fusion enzymes included a control with no poly(aspartic acid) tail (GACD0), a tail containing 5 aspartic acid residues (GACD5), and a tail containing 10 aspartic acid residues (GACD10). The purification, characterization, and polyelectrolyte precipitation of these enzymes has been published elsewhere [7].

β-galactosidase from *Escherichia coli* was used as the multimeric enzyme for the precipitation studies using pure enzymes. Three different enzymes were constructed, including a control with no poly(aspartic acid) tail (BGCD1), a tail containing 5 aspartic acid residues (BGCD5), and a tail containing 11 aspartic acid residues (BGCD11) (Zhao et al., 1990). The purification, characterization, and polyelectrolyte precipitation of these enzymes has been published elsewhere [8].

Zeta potential measurements were performed on β-galactosidase/PEI precipitates using a Lazer Zee Meter model 500, Zeta-Potential Instrument (Pen-Kem, Inc.). For these measurements, precipitation with PEI was carried out using
commercial (wild-type) β-galactosidase (WTBG) from the Sigma Chemical Co. WTBG was dialyzed to 100 mM NaOAc, pH 5.7, and adjusted to 0.50 mg/ml prior to complexation with PEI (55 kDa, Polysciences). PEI in 100 mM NaOAc, pH 5.7, was added to WTBG at various dosages. The final protein concentration was 0.25 mg/ml.
RESULTS AND DISCUSSION

Application of the model to precipitation studies will be discussed in two separate parts. The first section deals with monomeric enzymes which contain only one polypeptide tail, whereas the second section looks at tetrameric enzymes containing four tails. The system is defined with PEI as the macromolecule and the enzyme as the ligand. The amount of protein precipitated is taken as \([P]_{\text{Bound}}\). In order to compare the data to model predictions, the assumption had to be made that all of the PEI in the system was contained in the solid precipitate. This assumption has been shown to be fairly accurate with various polyelectrolytes up to the optimal dosage [3, 8, 12-14]. It then follows from Equation 1 that \(v\) is actually an apparent binding number based on the amount precipitated. The association constants obtained are then also apparent association constants for the same reason. For simplicity, however, the term 'apparent' will be dropped from the results and discussion which follow. It should, however, be kept in mind that the results are based on the observed precipitation and are not a true measure of the binding, i.e. they are only a measure of the binding which results in precipitation.

Application to Monomeric Enzymes: Glucoamylase

The precipitation curves obtained experimentally for the GACD fusion enzymes at the various ionic strengths studied are given in Figures 3a, 4a, and 5a. Also shown in these figures are the respective binding numbers calculated from Equation 1.

Maximum binding number

Determination of the maximum binding number, \(n\), was made by using Equation 1 to calculate values for \(v\) at various dosages. Binding plots were constructed by plotting the binding number, \(v\), versus the free protein...
Figure 3. The complexation of glucoamylase with PEI at $I = 20$ mM, pH 4.5: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data.
Figure 3. (continued) (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, GACD0; ▲, △, GACD5; ○, O, GACD10. The dashed line indicates an adjusted v for the tailed enzymes.
Figure 4. The complexation of glucoamylase with PEI at [I = 100 mM, pH 4.5: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data.
Figure 4. (continued) (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, GACD0; △, Δ, GACD5; ●, ○, GACD10. The dashed line indicates an adjusted v for the tailed enzymes.
Figure 5. The complexation of glucoamylase with PEI at I = 200 mM, pH 4.5: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data.
Figure 5. (continued) (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, GACD0; ▲, △, GACD5; ●, ○, GACD10. The dashed line indicates an adjusted v for the tailed enzymes.
concentration, [P], for the various fusion enzymes at each ionic strength, as shown in Figures 3b, 4b, and 5b. In contrast to the control enzyme, the binding curves for the tailed proteins show that v decreases at higher [P] rather than leveling off as expected. Typically, the binding number should increase at low [P] to a constant value at high [P] [15, 17]. The most likely explanation for the decrease in v upon increasing [P] is that not all of the polyelectrolyte is present in the precipitate. At least some of the polyelectrolyte remains in solution as soluble protein-polyelectrolyte complexes until nearly all of the protein has been precipitated. The polyelectrolyte concentrations in the solution could not be determined experimentally as a result of the extremely low amounts used in the precipitations. The sensitivity of the assay for PEI would have had an error of greater than 40% if all of the PEI were present in the solution and not in the precipitate.

Because of the decrease in v at higher [P], the maximum value obtained for v on each binding plot was taken as the maximum binding number for that enzyme at that ionic strength. Since the binding number was found to decrease with ionic strength, an absolute maximum binding number, n_{abs}, was determined by extrapolating n out to zero ionic strength. Figure 6 shows n_{abs} to be approximately 28 for all the versions of glucoamylase. This is considerably lower than the estimates of 60 and 300 found by the steric hindrance and electrostatic neutrality calculations, respectively. The higher maximum binding numbers observed for the tailed versions of the enzymes at the ionic strengths investigated are a result of the high charge density of the fusion tails being less susceptible to interference from ionic shielding than would the scattered charges on the surface of the protein.
Figure 6. Determination of the absolute maximum binding number for glucoamylase at pH 4.5: ■, GACD0; ▲, GACD5; ○, GACD10
Electrostatic cooperativity and intrinsic association constants

Cooperativity in binding was not observed for the precipitation of glucoamylase with branched PEI. As a result of the relatively constant binding numbers obtained for GACD0, Z was found to vary relatively little and therefore $\Phi(Z)$ could not be determined. As was evidenced by Clark and Glatz [3], the values of $|Z|$ were found to be quite high. Average values of Z for GACD0 were estimated to be 482, 636, and 710, at 20, 100, and 200 mM ionic strength, respectively. These high Z values indicate that there are an excess of unbound imine groups on the polyelectrolyte. This result agrees well with the hypothesis that branched PEI behaves as a compact sphere. The relatively large proteins would not have access to the internal charge of PEI. In the absence of cooperative binding, $K$ and $K_{int}$ become essentially equivalent and $K_{int}$ is therefore not analyzed separately.

Association constants

When combining $n_{abs}$ with Equation 6, a single value for the association constant $K_p$ was found to accurately describe the observed precipitation at each ionic strength for GACD0 (Figures 3a, 4a, and 5a). The predicted dependence of $K_p$ on I was found to qualitatively agree with experimental results in that $K_p$ decreases as the ionic strength is increased (Figure 7).

For both tailed enzymes, however, the binding number increases to a maximum and then decreases significantly at higher free protein concentrations, as can be seen in Figures 3b, 4b, and 5b. Again, this can be explained by the hypothesis that some PEI exists as soluble complexes with protein until nearly complete precipitation is obtained. Taking into account this possibility, $K_i$ values could be calculated by estimating the amount of polyelectrolyte involved in soluble complexes. This amount was determined by assuming the $\nu=n$ over the entire range.
Figure 7. Dependence of the association constants for glucoamylase on the ionic strength: ■, $K_p$; ▲, $K_t$ for GACD5 and GACD10.
of precipitation (dashed line on Figures 3b, 4b, and 5b), as was observed for the control protein. The amount of polyelectrolyte in the precipitating complex is then calculated by substituting \( n \) for \( v \) in Equation 1. Taking into account the amount of soluble PEI complexes, \( K_t \) values (Figure 7) for the precipitating complexes were calculated from fits to the experimental precipitation results (Figures 3a, 4a, and 5a) using the adjusted values for \( v \).

A constant value for \( K_t \) was found to accurately model the experimental results at each ionic strength (Figures 3a, 4a, and 5a). A single value for \( K_t \) was determined for the two fusion enzymes as a result of the nearly identical precipitation curves. As the theory predicted, \( K_t \) was found to decrease as \( I \) increases (Figure 7). Furthermore, \( K_t \) was found to be much greater than \( K_p \), indicating that the binding was dominated by tail-polyelectrolyte interactions.

As for the predicted behavior of \( K_r \), the theory could not directly account for the crossover in precipitation behavior of the tailed enzymes when compared to the control enzyme (see Figures 3a, 4a, and 5a). The tailed enzymes displayed only trace amounts of precipitation at low dosages, then precipitated to completion over a relatively narrow range of dosages. A possible explanation would be the presence of a critical dosage as is present in bridging theory [32-34]. At low dosages, the polyelectrolyte is saturated with the fusion protein and remains soluble. As the dosage is increased to the point where the polyelectrolyte begins to encounter other complexes before becoming completely saturated with protein, the complexes would begin to form bridges. The resulting increase in complex size and reduction in complex solubility would lead to precipitation.

The assumption that the polyelectrolyte is saturated with protein at low dosages is supported by the estimates of the maximum binding number. If all the
protein present in solution were to bind to the polyelectrolyte, the steric limit to binding \((v = 60)\) would be exceeded at dosages lower than 0.014. The experimental limit to binding \((v = 28)\) obtained by extrapolation of experimental data (Figure 6) would be exceeded at dosages lower than 0.030. These values do, in fact, cover the range where the presence of the tails was found to increase the solubility of the complex (Figures 3a, 4a, and 5a).
Application to Multimeric Enzymes: β-galactosidase

The precipitation curves obtained experimentally for the BGCD fusion enzymes at the various ionic strengths studied are given in Figures 8a, 9a, and 10a. Also shown in these figures are the respective binding numbers calculated from Equation 1.

Maximum binding number

The binding curves obtained for the β-galactosidase fusion proteins reveal much lower binding numbers than were obtained for glucoamylase. The binding curves are shown in Figures 8b, 9b and 10b. These lower binding numbers result from the greater size of β-galactosidase (470 kDa versus 65 kDa) which would decrease the number of enzymes able to bind to a polyelectrolyte molecule. At a constant ionic strength, the binding numbers were found to be constant for the control protein (BGCD1), and decreased only slightly at higher [P] for the tailed proteins. An unexpected effect was encountered with the dependence of n on I. As I was increased from 20 mM to 100 mM, n actually increased for both of the fusion tail enzymes (Figure 11). The untailed version displayed the expected decrease in n upon increasing I. The value of nabs for β-galactosidase was determined to be 10 from Figure 11. This value for n is considerably less than the estimates of 20 and 28 from the steric limitation and electrostatic neutrality calculations, respectively.

Electrostatic cooperativity

Cooperativity as a result of electrostatic interactions was not evidenced for the precipitation of β-galactosidase with branched PEI. No correlation between the net complex charge and the binding constants could be determined as the result of a relatively constant binding number, which in turn yields a relatively constant
Figure 8. The complexation of β-galactosidase with PEI at I = 20 mM, pH 5.7: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data.
Figure 8. (continued) (b) dependence of the binding numbers on the free protein concentration, [P]; □, □, BGCD1; △, Δ, BGCD5; ●, ○, BGCD11
Figure 9. The complexation of β-galactosidase with PEI at $I = 100$ mM, pH 5.7: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data.
Figure 9. (continued) (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, BGCD1; ▲, △, BGCD5; ○, O, BGCD11
Figure 10. The complexation of β-galactosidase with PEI at I = 200 mM, pH 5.7: (a) effect of dosage on the precipitation (closed symbols, solid line) and binding numbers (open symbols, dashed line). The solid lines shown for the precipitation curves are fits by the model to the data.
Figure 10. (continued) (b) dependence of the binding numbers on the free protein concentration, [P]; ■, □, BGCD1; ▲, △, BGCD5; ○, O, BGCD11
Figure 11. Determination of the absolute maximum binding number for β-galacto-sidase at pH 5.7: ■, BGCD1; ▲, BGCD5; ○, BGCD11
complex charge. High average Z values were once again observed: 534, 549, and 577 for 20, 100, and 200 mM ionic strength, respectively.

**Association constants**

As was stated previously, we hypothesize that the high charge density tails act as selective electrostatic interaction sites which enable multiple polyelectrolytes to bind per enzyme. Since increasing amounts of polyelectrolyte would lead to larger complex sizes, the formation of the matrix should be dependent on the dosage. Association constants were calculated using Equation 6 and the apparent v from Equation 1. It was found that ln K_p and ln K_t both displayed a linear dependence on the dosage (Figures 12, 13, and 14) and hence can be described as

\[ K = K_0 e^{mD} \]  

(22)

with an intercept of ln K_0 and a slope of m. Fitted curves to the data, shown in Figures 8a, 9a, and 10a, were calculated using Equation 5 and association constants from linear fits of ln K_p and ln K_t versus D.

The dependence of K_p on I follows the expected trend for polyelectrolyte precipitation without matrix formation from Equation 15: K_p decreases as I increases (Figure 12). For the fusion tails, however, K_t increases upon increasing ionic strength (Figures 13 and 14). This is understandable if the increase in I is not sufficient to disrupt protein-polyelectrolyte binding but can reduce the electrostatic barrier to formation of a matrix of complexes carrying a net charge of Z. This could well describe the situation for the fusion enzymes, which have shown strong binding on a diethylaminoethyl ion exchange column [8]. In those experiments, also conducted at pH 5.7, ionic strengths of 0.55, 0.61, and 0.68 were
Figure 12. Effect of dosage and ionic strength on the BGCD1 association constant, pH 5.7: ■, I = 20 mM; ▲, I = 100 mM; ○, I = 200 mM
Figure 13. Effect of dosage and ionic strength on the BGCD5 association constant, pH 5.7: ■, I = 20 mM; ▲, I = 100 mM; ○, I = 200 mM
Figure 14. Effect of dosage and ionic strength on the BGCD11 association constant, pH 5.7: ■, I = 20 mM; ▲, I = 100 mM; ●, I = 200 mM
required to elute the BGCD1, BGCD5, and BGCD11, respectively. These ionic strengths are all higher than those investigated in the precipitation studies.

An increase in precipitation upon increasing the ionic strength can also be found in analogous situations where two highly charged components form complexes: the precipitation of polyelectrolytes by polyelectrolytes [35] or the flocculation of silica with PEI [20]. In both cases, the increase in precipitation with ionic strength was said to be the result of an increase in ionic shielding which decreased the electrostatic repulsion between the protein-polyelectrolyte complexes.

Net charge considerations

Although the net charge of an enzyme is a good indication as to the degree of interaction with polyelectrolytes, localized charges also play a significant role. The extent to which the potential of these charges extends into solution is given by the thickness of the double layer [36]. The thickness of the double layer was calculated to be 22, 9.6, and 6.8 Å at 20, 100, and 200 mM ionic strength respectively. Although the net charge of the complexes was calculated to be positive, the exterior charge which is available for interaction should be negative as a result of the relatively small thickness of the double layer, when compared to the size of the protein, if the polyelectrolyte is surrounded by the negatively charged protein. As is shown in Figure 15, the zeta potential of the flocs at 100 mM ionic strength is negative at low dosages where the polyelectrolyte is saturated by protein, indicating that the interacting surfaces of the flocs indeed does have a negative character. As the dosage is raised past the point where the protein is completely removed from solution, the zeta potential increases to become positive. This is consistent with the hypothesis that the positively charged polyelectrolyte continues to bind to the exterior of the complexes after all the protein has been incorporated into the flocs.
Figure 15. Effects of the polyelectrolyte dosage and the percent protein precipitated on the zeta potential for commercial β-galactosidase in pH 5.7, 100 mM NaOAc.
Formation of the complex

Figures 12, 13, and 14 show that $K_p$ and $K_t$ increase as the dosage increases. That the slope for the control enzyme was not zero may be attributed to the fact that the control enzyme contains one negatively charged aspartic acid residue per fusion tail. It is therefore not a true control, but rather possesses a 'minimal' tail and would be capable of matrix formation.

The minimum dosage which would be necessary for gel formation can be estimated by setting $p_f = 1$ in Equation 21 if the measured $n_{abs}$ is the true limit to binding. However, the steric estimates of the maximum binding number were considerably higher. Setting $p_f = n_{\text{experimental}}(n_{\text{steric}})^{-1}$, which yields $p_f = 0.5$, would be a measure of the saturation actually realized. Estimates of the dosages which would result in the formation of a gel are given in Table 2 for two values of $g$. Any dosage greater than these values should result in the formation of a gel and therefore precipitation of the complex. These minimum values for $D$ demonstrate the validity of using the matrix formation theory to estimate solubility criteria since precipitation was observed for $\beta$-galactosidase at $D > 0.002$.

<table>
<thead>
<tr>
<th>$p_f$</th>
<th>$g=2$</th>
<th>$g=4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.0026</td>
<td>0.0017</td>
</tr>
<tr>
<td>0.5</td>
<td>0.00015</td>
<td>0.00010</td>
</tr>
</tbody>
</table>
CONCLUSIONS

A model based on multiple equilibrium binding has been developed which qualitatively predicts the polyelectrolyte precipitation of enzymes possessing charged fusion tails. The model incorporates a separate affinity to account for the binding of the charged tail to the polyelectrolyte. Electrostatic cooperativity is not evidenced for the binding of negatively charged proteins to branched PEI.

For the monomeric glucoamylase, the maximum number of enzymes which could bind per PEI molecule was found to be approximately 28, which is significantly lower than the steric estimate of 60. Both $K_p$ and $K_f$ were found experimentally to follow the model predictions: $K_p$ and $K_f$ decrease as the ionic strength is increased. $K_f$ was found to be much greater than $K_p$, indicating that binding was dominated by the charged tails of the fusion enzymes.

For the multimeric β-galactosidase, which has a molecular weight approximately 8-fold that of glucoamylase, the absolute maximum binding number was found to be approximately 10. This is significantly lower than the steric estimate of 20. The binding number of the tailed enzymes actually increased with increasing ionic strength. It was found that this behavior could be understood if the precipitate is viewed as an interconnected matrix with the multiple fusion enzymes strongly binding to and crosslinking multiple polyelectrolytes. Values for $K_p$ were found to decrease as I increased, whereas values for the $K_f$ of both fusion enzymes was found to increase. The apparent increase in the $K_f$ values is proposed to be due to reduced intermolecular repulsion in the tightly bound matrix upon increasing I. Both $K_p$ and $K_f$ were found to increase with increasing dosage.
ACKNOWLEDGMENTS

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REFERENCES


GENERAL CONCLUSIONS

Fusions of negatively charged poly(aspartic acid) tails to β-galactosidase and glucoamylase as well as positively charged poly(arginine) tails to β-galactosidase have been shown to result in enhanced separation behavior over that of the control or native enzymes. In precipitations from crude extracts of poly(aspartic acid) tailed β-galactosidase using polyethyleneimine, interference from nucleic acids was experienced. Precipitations of poly(arginine) tailed β-galactosidase using poly(acrylic acid) experienced no such interference, yet no activity could be recovered from the precipitates. No precipitation of glucoamylase could be obtained from the supernatant of the fermentation using polyethyleneimine. This was most likely due to interfering components present in the supernatant, since precipitation could be achieved from diafiltered supernatants.

Precipitations of purified poly(aspartic acid) tailed enzymes were conducted for the purpose of developing a model for the precipitation of genetically altered proteins containing charged fusion tails. The precipitations themselves yielded some interesting results. The monomeric glucoamylase fusion enzymes displayed the expected behavior: the fusion tails resulted in enhanced precipitation and increasing ionic strength decreased the amount of precipitation at a given polyelectrolyte dosage for both control and fusion tailed enzymes. Wild-type and control tetrameric β-galactosidase also displayed this behavior. For the fusion tailed β-galactosidase, the presence of the tails still resulted in enhanced precipitation, yet the amount of precipitation at a given polyelectrolyte dosage actually increased upon increasing ionic strength.

A model of polyelectrolyte precipitation has been developed to account for the presence of the charged fusion polypeptides based on multiple equilibria binding
with cooperativity effects and multiple association constants. The model treats the enzyme and the fusion tail as having separate association constants. Cooperativity as a result of electrostatic effects was not observed in these precipitations. Experimental results for the monomeric enzyme correlate well with model predictions. For the tetrameric enzyme, an additional factor in the cooperativity of binding was added to account for the formation of a tightly bound matrix. It is proposed that the formation of an interconnected matrix results from the multiple tails on an enzyme strongly binding to multiple polyelectrolytes. The solubility of the tightly bound protein-polyelectrolyte matrix would decrease upon increasing I as a result of increased ionic shielding which reduces the intermolecular repulsion.
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APPENDIX A: NET ESTIMATED CHARGE OF ENZYMES

For approximations as to precipitation behavior, an estimation of the net protein charge at various pH values is necessary. The estimations were calculated using the Henderson-Hasselbalch equation:

\[ \text{pH} = \text{pK} + \log \left( \frac{[R^-]}{[RH]} \right) \]  
(A.1)

where \( RH \leftrightarrow R^- + H^+ \), or

\[ \text{pH} = \text{pK} + \log \left( \frac{[R]}{[RH^+]} \right) \]  
(A.2)

where \( RH^+ \leftrightarrow R + H^+ \).

The fraction of dissociation for each amino acid was calculated and summed to yield the net charge due to each type of amino acid at a given pH. The fraction of dissociation is calculated from:

\[ \frac{[R]}{[RH]} = 10^{(\text{pH}-\text{pK}_n)} = \frac{x}{n-x} \]  
(A.3)

which yields

\[ x = n \frac{10^{(\text{pH}-\text{pK}_n)}}{1+10^{(\text{pH}-\text{pK}_n)}} \]  
(A.4)

where \( n \) is the number of the type of amino acid present in the protein and \( x \) is the number of those amino acids in dissociated form.

This calculation was performed for each type of amino acid and summed to yield a net charge on the protein. Estimates of pK values (Stryer, 1988) and the number of ionizable amino acids in the original enzymes are given in Table A.1.
Table A.1: Estimates of the ionizable amino acid pK values from Stryer (1988) and the number of each type of amino acid present in the unmodified enzymes

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>pK</th>
<th>WTBG</th>
<th>GACD0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>6.5</td>
<td>136</td>
<td>4</td>
</tr>
<tr>
<td>Lysine</td>
<td>10</td>
<td>80</td>
<td>8</td>
</tr>
<tr>
<td>Arginine</td>
<td>12</td>
<td>264</td>
<td>15</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>10</td>
<td>124</td>
<td>18</td>
</tr>
<tr>
<td>Cysteine</td>
<td>8.5</td>
<td>64</td>
<td>7</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>4.4</td>
<td>256</td>
<td>35</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>4.4</td>
<td>248</td>
<td>18</td>
</tr>
<tr>
<td>Carboxy-Terminus</td>
<td>3.1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Amino-Terminus</td>
<td>8</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

These amino acid counts were obtained from the amino acid sequence of each of the enzymes. The β-galactosidase amino acid sequence is from Kalnins et al. (1983). The glucoamylase amino acid sequence is from Nunberg et al. (1984). Additional ionizable amino acids present in the tailed versions of the enzymes are given in Table A.2. The dependence of charge on pH for the various fusions of β-galactosidase from *E. coli* and glucoamylase from *A. niger* are shown Tables A.3 and A.4, respectively. Boxes indicate the charge estimates at the pH of experimentation for the various fusion proteins.
Table A.2: Number of additional ionizable amino acids present in the fusion enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Arginine</th>
<th>Tyrosine</th>
<th>Cysteine</th>
<th>Aspartic Acid</th>
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<tr>
<td>BG290</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>BGCD5</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>20</td>
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<tr>
<td>BGCD11</td>
<td>0</td>
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<td>44</td>
</tr>
<tr>
<td>BGCD16</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>BGCR0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>(BGCD1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BGCR5</td>
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<td>0</td>
<td>5</td>
</tr>
<tr>
<td>GACD10</td>
<td>0</td>
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Table A.3: Estimates of the enzyme charge at various pH values for β-galactosidase

<table>
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<tr>
<th>pH</th>
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<th>BG290</th>
<th>CR15</th>
<th>CR10</th>
<th>CR5</th>
<th>CR0</th>
<th>CD1 or CD11</th>
<th>CD5</th>
<th>CD16</th>
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<td>5.0</td>
<td>73.0</td>
<td>73.8</td>
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<td>109.8</td>
<td>89.8</td>
<td>69.8</td>
<td>57.1</td>
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<td>21.9</td>
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<tr>
<td>5.1</td>
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<td>55.3</td>
<td>111.3</td>
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APPENDIX B: ATTEMPTS AT ISOLATING β-GALACTOSIDASE FUSION PEPTIDES

In order to verify the integrity of the tails on the β-galactosidase fusion proteins, it was initially decided to attempt to cleave the tails with CNBr and subsequently sequence the purified peptides. It was for this purpose that the methionine and tyrosine residues were originally included in the fusion peptide sequence. CNBr cleaves proteins at the carboxyl terminus of methionine, an amino acid which is relatively rare in most proteins (Needleman, 1970). The tyrosine residue was added to facilitate detection of the fusion peptide by absorption at 280 nm.

Cleavage of the tail by CNBr would be advantageous since it is a relatively simple method of cleavage, the tail methionine is readily accessible on the surface of the protein, and the cleavage of internal methionine peptide bonds should be lower since peptide fragment yields for the CNBr cleavage of β-galactosidase have been reported to be as low as 25% (Cahnmann et al., 1966; Plaxton and Moorhead, 1989; Fowler, 1978; Rhee, 1990; Steers et al., 1965). The matter of obtaining the fusion tail in pure form is complicated by the fact that β-galactosidase from E. coli contains 23 methionine residues per monomeric subunit (Silhavy and Berman, 1984). The method also suffers from the occurrence of unspecific cleavage reactions (Schreiber and Witkop, 1964; Allen, 1989; Fowler, 1978).

Attempts were also made to cleave the aspartate-proline linkages by acidic cleavage. This method would result in only 5 fragments as opposed to 25 fragments for complete cleavage using CNBr. Again, the cleavage method suffers from undesired side reactions and variable yields (Smith, 1981; Landon, 1977).
Materials and Methods

Cleavage was performed on lyophilized samples of affinity-purified enzymes. Final concentrations of β-galactosidase in the cleavage reaction solutions ranged between 1.0 and 3.0 mg/ml. Standard procedures (Robyt and White, 1987) were among the various cleavage conditions attempted for the CNBr and acidic cleavage of proteins. For the standard CNBr cleavages, CNBr (Pierce) was dissolved in 70% formic acid and then added to the lyophilized enzyme at room temperature. Final molar ratios of CNBr to methionine ranged between 50 and 150. The solutions were then capped with Parafilm and allowed to react. After one to six hours reaction time, the solutions were shell-frozen in an acetone-dry ice bath and lyophilized for six hours. All of the above steps involving CNBr were performed in a ventilation hood using gloves, due to the extreme toxicity of CNBr. Higher pH values of 5.0, 6.0, and 7.0 were also investigated using 0.1 M sodium phosphate in an attempt to avoid denaturation of the enzyme. Otherwise the experimental procedure was identical to that using formic acid.

Acidic cleavage was performed using 70% formic acid. The acid was added to lyophilized β-galactosidase, capped with Parafilm, and placed in a 40°C water bath for 48 hours. Samples were then either shell-frozen and lyophilized for six hours, or directly analyzed by HPLC.

Attempts to purify the fusion peptides were made using HPIEC, HPGPC, and reverse-phase chromatography. HPIEC and HPGPC columns and procedures used to analyze the samples followed those given in Paper II. HPGPC analysis was also performed using 6.0 M guanidine HCl (Kato et al, 1980) on a Beckman TSK3000SW column. For reverse phase chromatography, an Aquapore RP-300 column was used. The reverse phase buffer was 0.1% TFA with a gradient to 70%
acetonitrile and 0.1% TFA over 30 minutes at a flow rate of 1.0 ml/min. In all cases, injections ranged from 30 μg to 500 μg total protein. Various proteins and amino acids were used as references in 1.0 mg/ml stock solutions. Detection of the fragments was performed using ultraviolet absorption at 220 and 280 nm. Detection at 280 nm was used to follow the tyrosine residue on the tail peptide. The additional detection at 220 nm was used after calculations showed that this wavelength would be more sensitive to the detection of the fusion peptide (Robyt and White, 1987). Lower wavelengths could not be used since excessive absorbance from the buffer itself was encountered.

**Results and Discussion**

Efforts to cleave β-galactosidase fusions were unsuccessful in that the fusion peptide could not be isolated. For CNBr cleavage in 70% formic acid, analysis by HPSEC showed that β-galactosidase lost its tetrameric structure and was therefore inactivated within one minute. The loss of activity was confirmed using the ONPG assay (Paper II). No further cleavage was noted after two hours. The times of cleavage were seen to be similar to the results stated in the literature. The rate of cleavage was found to be comparable in 0.1 M sodium phosphate. For acidic cleavage without CNBr, the time of the reaction was much longer. The reaction could therefore be tracked by HPLC analysis. Figure B.1 shows just such an experiment. The β-galactosidase peak can be seen to disappear within 24 hours. β-galactosidase activity was lost upon contact with the acidic solution.

Attempts using chromatographic techniques to isolate the peptide fragments were unsuccessful. Analysis by gel permeation and ion exchange chromatography did not reveal any peaks at the expected elution times for the fusion peptides. Concentrating the samples approximately 10-fold prior to analysis did not alter the
Figure B.1 HPLC analysis of the acidic cleavage of β-galactosidase over time
results. That no tail peptides could be isolated may be attributed to the difficulties associated with the low yields of the cleavage reactions and any accompanying side reactions which can result in nonspecific cleavages. Final yields as low as 7% have been reported in the isolation of certain β-galactosidase fragments (Fowler and Zabin, 1981). Furthermore, the amounts of fusion β-galactosidase available for cleavage were on the order of two or three magnitudes lower than the amounts of protein used in the literature.

Further experiments were proposed yet not attempted since it was decided that sufficient evidence had been obtained to verify the presence and integrity of the tails. These experiments included the purchase of aspartic acid polypeptides of specific lengths between 5 and 20 residues for use as standards in HPLC analyses, analysis of the total amino acid content to determine relative amounts of aspartic acid, and specific cleavage by proteases followed by isolation and amino acid sequencing of the polypeptide containing the fusion tail.
APPENDIX C: GACD PRECIPITATIONS

Glucoamylase cleaves α-(1→4) and α-(1→6) glycoside bonds from the non-reducing ends of starch to yield β-D-glucose. The enzyme is used both in the production of glucose from starch and as an intermediate in the production of high fructose corn starch. Glucoamylase is produced by a wide range of organisms, most of which are filamentous fungi. The enzyme chosen for this study is produced by *Aspergillus awamori* and exists in two forms, GAI and GAII. These forms differ in their ability to hydrolyze raw starch. The difference is due to the presence of a region at the carboxyl end of GAI, which GAII lacks. The region is approximately 17 kDa, including amino acids 513 to 616, and is believed to be removed through limited proteolysis of GAI (Svensson et al., 1986). The region enables GAI to bind to raw starch.

The focus of this study was to enhance the selectivity of polyelectrolyte precipitation through the genetic modification of glucoamylase. This study was meant to expand upon the results obtained previously using β-galactosidase (Parker et al., 1990; Zhao et al., 1990), a large, intracellular tetrameric enzyme. Glucoamylase is a small, monomeric secreted enzyme which is being expressed in *Saccharomyces cerevisiae* (Innis et al., 1985). An active, shortened version of glucoamylase was genetically modified to contain carboxyl terminal poly(aspartic acid) peptides of various lengths. Polyelectrolyte precipitations were conducted using primarily 55 kDa polyethyleneimine (PEI), the same polyelectrolyte used to study the precipitation of fusions with β-galactosidase. Precipitations were performed on the fermentation supernatant, diafiltered supernatant, and affinity-purified enzymes. The reader is referred to Suominen et al. (1992) for a more comprehensive overview of the work performed thus far with various glucoamylase
fusions. This appendix is a summary of the work conducted solely by myself and includes results used for modeling in Paper III.

Materials and Methods

Enzyme construction and production

Genetic modification of the enzyme had been carried out previously in order to investigate the active site of the enzyme (Sierks, 1988). The same gene was modified to create the carboxyl terminal fusions of glucoamylase used in this study. The gene is a cDNA clone from *Aspergillus awamori*. It is contained on the pGAC9 plasmid (Innis et al., 1985), a gift from the Cetus corporation (Emeryville, CA). The base gene for the carboxyl terminal fusions encodes the first 485 amino acids. The fusion tails were constructed by replacing the 3' 0.55 kb BamHI-HindIII fragment of the glucoamylase gene with synthetic BamHI-HindIII restriction fragments encoding 0, 5, and 10 aspartic acid residues. The synthetic restriction fragments were constructed at the Iowa State Nucleic Acid Facility. The fusion enzymes are designated GACD0, GACD5, and GACD10, respectively. The notation is: GA = glucoamylase, C = carboxyl terminal fusion, D = aspartic acid, and # = number of residues. The resulting carboxyl terminal fusions are depicted in Table C.1. The enzymes were grown in *S. cerevisiae* C468 (Innis et al., 1985) in SD medium (Sherman et al., 1983) at 30°C supplemented with tryptophan for the auxotrophic mutation of the strain.

Purification

GACD fusion proteins were purified from 20-fold concentrated and diafiltered culture supernatants (0.1M sodium acetate, pH 4.5, Amicon S1 spiral cartridge ultrafiltration unit, MWCO 10 kDa). The concentrate was loaded into an
acarbose-Sepharose affinity column (Clarke and Svensson, 1984). Elution was achieved using 1.7 M Tris-HCl, pH 7.6. The eluted fractions containing glucoamylase activity were pooled and dialyzed to 20 mM sodium acetate at 4°C.

Table C.1. The sequences of the fusion tails at the carboxyl terminus of glucoamylase (Suominen et al., 1992). The restriction sites, BamHI and HindIII, used to insert the DNA cassettes are underlined. Both the genetic and protein sequences are given. The *** indicates a stop codon which terminates protein translation.

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PEI precipitations

Precipitations were performed on supernatants, diafiltered supernatants, and purified enzymes. Poly(ethylene imine) (PEI) of 55 kDa as a 50% wt/vol solution in water from Aldrich Chemical Co. (Milwaukee, WI) was made to 20 μg/ml stock solutions in the appropriate buffer. Poly(diallyldimethyl ammonium chloride) (PDADMAC) of 240 kDa as a 20% wt/vol solution in water from Polysciences (Warrington, PA) was made to 200 μg/ml stock solutions in the appropriate buffer. Precipitations of purified enzymes were conducted using 20 μg/ml enzyme in total volumes of 400 μl with varying dosages of polyelectrolyte. The mixtures were immediately vortexed for 15 seconds, incubated at room temperature for 15
minutes, and centrifuged in a microcentrifuge at 12,000 g for 15 minutes. In the precipitation of supernatants and diafiltered supernatants, a total of 50 μg in 1 ml final volume was used. This concentration was approximately equal to the concentration in the fermentation supernatants prior to any purification. The glucoamylase activity and protein content of the supernatant were immediately determined. In some cases, the pellets were resolubilized by vortexing in 1.0 M sodium acetate buffer, pH 4.5, and assayed for glucoamylase activity.

**Glucoamylase and protein assays**

Glucoamylase activity was assayed at 30 °C using 2 % soluble starch (in 0.05 M acetate buffer, pH 4.5) as the substrate. The glucose produced from the starch was measured using the glucose oxidase assay of Banks and Greenwood (1971). A unit of glucoamylase activity was defined as the μmol of glucose liberated per minute under reaction conditions. Total protein content was determined using the Pierce BCA Protein Assay with bovine serum albumin (Pierce Chemicals) as the protein standard.

**Results and Discussion**

**Precipitation of purified enzymes**

For purified enzyme solutions, precipitations were carried out with PEI in 20, 100, and 200 mM sodium acetate, pH 4.5. The results can be seen in Figures 3a, 4a, and 5a contained in Paper III. The expected decrease in precipitation at a given dosage is observed upon increasing ionic strength for all the fusion proteins. A notable difference in precipitation behavior is observed between the fusion enzymes and the control enzyme (GACD0). Both GACD5 and GACD10 display a lesser
tendency to precipitate than GACD0 at lower PEI dosages, then a higher tendency to precipitate at higher dosages.

A possible explanation for this behavior would be that the tails possess a relatively high affinity for the polyelectrolyte and would thus orient the enzyme with the tail facing the polyelectrolyte. The result at low dosages, where the polyelectrolyte is saturated with protein, the complexes would most likely be soluble. As the dosage is increased to the point where the polyelectrolyte begins to encounter other complexes before becoming completely saturated with protein, the complexes would begin to form bridges (Hogg, 1984; LaMer and Healy, 1963a; LaMer and Healy, 1963b). The resulting increase in complex size and reduction in complex solubility would lead to enhanced precipitation. This theory is supported by the binding curves obtained for GACD0, as seen in Figures 3b, 4b, and 5b contained in Paper III. The protein comprises a higher portion of the complex (higher v) at lower polyelectrolyte dosages (higher [P]).

Notable is the fact that there is no significant difference in the precipitation behavior between GACD5 and GACD10. This indicates that the addition of aspartic acid residues in the polypeptide tail beyond 5 residues is of no further benefit. The optimal tail length for these monomeric fusion enzymes is therefore five residues. The estimated net charges of the fusion enzymes, as given in Table A.4, shows that at pH 4.5, the addition of five aspartic acid residues more than doubles the net charge of the enzyme. While the addition of a further five charged residues increases the net charge a proportional amount, it evidently offers no further advantage over the high charge density already present in GACD5. The difference in precipitation between the fusion enzymes and GACD0 becomes greater as the ionic strength is increased. This indicates that the fusion tail increases the strength
of binding to the polyelectrolyte. The resolubilization of all the enzymes at the highest dosage for 20 mM ionic strength is typical of precipitations with polyelectrolytes of the molecular weight used in this study (Shieh, 1989; Clark, 1988; Hidalgo and Hansen, 1969)

Precipitation of diafiltered supernatants

For diafiltered supernatants, precipitations with PEI at pH 4.5 resulted in incomplete precipitation over the entire range of precipitation, as seen in Figure C.1. The observed trend of precipitation was similar to that of purified enzymes in that GACD 5 and GACD10 behaved nearly identically. The enhancement in precipitation over that of GACD0 was greater than was observed for the purified enzymes. This may be a result of the ability of the fusion tails to overcome interference from other components due to their higher affinity for the polyelectrolyte.

Recovery of activity from the precipitates was found to be nearly complete for all the enzymes (Figure C.1). Some losses were experienced during centrifugation, even in the absence of polyelectrolyte, perhaps as a result of adsorption. These losses would account for nearly all the shortfall in recovery.

Attempts were made to raise the maximum amount precipitated by using a strong polyelectrolyte instead of the weak polyelectrolyte PEI. A strong polyelectrolyte would have the advantage that its degree of ionization would remain constant over a wide pH range, whereas the degree of ionization of a weak polyelectrolyte is pH dependent. Precipitations with the strong polyelectrolyte PDADMAC at pH 4.5 resulted in maximum precipitation of approximately 30% (data not shown). Upon raising the pH to 6.0, the maximum amount precipitated increased to approximately 80% (Figure C.2). At this pH, however, the difference in
Figure C.1. Precipitation (solid symbol) and recovery of activity from the precipitate (open symbol) for the complexation of glucoamylase with PEI in diafiltered supernatant at pH 4.5: ■, □, GACD0; ▲, △, GACD5; ○, O, GACD10
Figure C.2. The precipitation of glucoamylase from diafiltered supernatant with PDADMAC (solid) and PEI (open) at pH 6.0: ■, GACD0; ▲, GACD5; ○, O, GACD10
precipitation behavior between the fusion enzymes nearly disappeared. This is most likely due to the higher net charge on the enzymes at this pH (see Table A.4). Precipitation with PEI at pH 6.0 resulted in a maximum of only 30% precipitated for GACD10 (Figure C.2). This can be explained by the fact that the degree of ionization of PEI decreases as the pH is increased.

Precipitation of supernatants

Polyelectrolyte precipitations of glucoamylase from untreated supernatants were unsuccessful. Attempts at precipitation included the use of PEI and PDADMDAC over wide dosage ranges (0.0005 to 20 g/g) at pH values of 4.5, 5.5, and 6.0. No precipitation was observed under any of these conditions. It is thought that components present in the culture supernatant may be responsible for the interference in precipitation. Total protein content did not change under any of the precipitation conditions, indicating that no precipitation of other proteins was occurring to any significant degree.

However, upon adding β-galactosidase (Sigma Chemicals) to the supernatant at pH 6.0, complete precipitation of β-galactosidase could be obtained at 0.2 g/g dosage. This would seem to indicate that the binding of glucoamylase to the polyelectrolytes is weaker and therefore disrupted in the presence of other constituents in the supernatant.

Conclusions

This study has expanded the results confirming the enhancement of polyelectrotye precipitation using genetic fusions of charged polypeptides to proteins. It has been shown that the precipitation of a smaller, secreted, monomeric, and glycosylated enzyme can be enhanced in this manner. An optimal tail length of 5 aspartic acid residues was observed, beyond which no further enhancement in
precipitation is found. This is less than what was observed in precipitations with fusion
tailed β-galactosidase where an optimal tail length of approximately 10 residues
was found (Niederauer et al., 1993; Zhao et al., 1990; Parker et al., 1990).

Addition of polyelectrolytes to untreated supernatants containing the fusion
enzymes resulted in no precipitation. However, upon supplementing the
supernatant with wild-type β-galactosidase and precipitating at pH 6.0, complete
precipitation of β-galactosidase could be achieved. It is thought that the binding of
glucoamylase to the polyelectrolyte is relatively weak and therefore disrupted in the
presence of other constituents in the supernatant. This is supported by the fact that
precipitation could be achieved in diafiltered supernatants. Overall, these results
suggest that charged polypeptides can be genetically fused to a variety of proteins
to enhance their separation by polyelectrolyte precipitation.
APPENDIX D: DERIVATION OF $\Phi(Z)$ AND $K_{\text{INT}}$

To evaluate the functionality of $\Phi(Z)$, Tanford (1961) proposed that it is directly related to the change in electrostatic free energy of the complex, $\Delta G_{\text{el}}$. He considered the process of adding an average of $dn$ ligands of charge $Z_p$ to a mole of macromolecule. On the average, $dv/n$ is added to each one of the identical sites of the macromolecule upon ligand binding. The resulting free energy change per mole of sites, $dG$, is then given by the product of Equation 10 from Paper III and $dv/n$:

$$dG = [\Delta G^o + RT\ln K] \frac{dv}{n}$$

(D.1)

Tanford (1961) considered this change as the result of four successive steps, each of whose free energy change was evaluated in turn. The first step involves discharging the macromolecule, which results in a free energy change per mole of sites, $dG_1 = -\Delta G_{\text{el},x}(Z)/n$. Secondly, the ligands to be complexed must be discharged. Per mole of sites, $dv/n$ moles of ligands require a free energy change to remove the ligand charge, $dG_2 = -\Delta G_{\text{el},p}dv/n$. Combination of the sites on the discharged macromolecule and ligands requires a free energy change of $dG_3 = (\Delta G^* + RT\ln K)dv/n$. Here, $\Delta G^*$ is the standard free energy change for the reaction of discharged macromolecule and ligand, which may differ greatly from that for the reaction between charged species. The final step involves restoration of the net charge. The charge now belongs to the macromolecule-ligand complex and is given by $(Z + Z_pdv)$. Per site the charge is $(Z + Z_pdv)/n$, resulting in a free energy change for this step of $dG_4 = \Delta G_{\text{el},x}(Z + Z_pdv)/n$, which can be rearranged to yield $dG_4 = [\Delta G_{\text{el},x}(Z) + (\partial\Delta G_{\text{el}}/\partial Z)Z_pdv]/n$. An additional step was provided by Clark and Glatz (1991) for the phase change resulting in a solid precipitate. The associated free energy change is $dG_5 = -\Delta G_{\text{cav}}dv/n$, where $\Delta G_{\text{cav}}$ is the free energy change required for the formation of a cavity in the solvent equal to the volume of the solid
phase being created. The negative sign is a result of the closing of the cavity upon removal of the macromolecule-ligand complex from the solvent.

Summing dG₁ through dG₅ yields the overall free energy change per mole of sites for the combination of dn moles of ligands with one mole of macromolecule to form a precipitate:

\[
dG = \left[-\Delta G_{el,p} + \Delta G^0' + RT\ln K + \left(\frac{\partial \Delta G_{el}}{\partial Z}\right)Z_p - \Delta G_{cav}\right] \frac{dv}{n}
\] (D.2)

Upon comparison with Equation D.1 the standard free energy change is seen to be

\[
\Delta G^0 = -\Delta G_{el,p} + \Delta G^0' - \Delta G_{cav} + \left(\frac{\partial \Delta G_{el}}{\partial Z}\right)Z_p
\] (D.3)

In order to determine the functionality of \( \Phi(Z) \), assumptions need to be made about \( \Delta G_{el}(Z) \). In the development of their model, Clark and Glatz (1991) used a modification of the Debye-Huckel theory by Melander and Horvath (1977) for the free electrostatic energy of the macromolecular complex. It was used because of its applicability to a wide range of solution ionic strengths. Melander and Horvath combined the Debye-Huckel theory for a protein ion, which is accurate at low ionic strengths, with Kirkwood's expression (1939), which is appropriate at high ionic strengths, to yield the general formula

\[
\Delta G_{el} = \frac{Z_i^2}{2} \left( A - \frac{B\sqrt{I}}{1 + C\sqrt{I}} \right) - \Delta IR T
\] (D.4)

in which the macromolecule is modeled as an impenetrable sphere with the net charge distributed evenly over the surface. The latter part of Equation D.4 is a modification of the Debye-Huckel theory using Kirkwood's theory for ion-dipole interactions. Basing \( \Delta G_{el}(Z) \) Equation D.4 offers the advantage that \( \partial \Delta G_{el}/\partial Z \) is zero for zero net charge, yielding
\[ \Delta G^\circ_{\text{int}} = -\Delta G_{\text{el},p} + \Delta G^\circ - \Delta G_{\text{cav}}. \]  

Additionally, since for the case of zero net charge it is assumed that \( Z = 0 \), one obtains \( \Delta G^* = \Delta G^*_{\text{int}} \) from Equation 10 from Paper III. Upon comparison of Equations D.3, D.5, and 10 from Paper III, it can be resolved that

\[ \Phi(Z) = \frac{\left( \frac{\partial \Delta G_{\text{el}}}{\partial Z} \right)_{Z_p}}{RT} \]  

The form of \( K_{\text{int}} \) may now also be determined since

\[ RT \ln K_{\text{int}} = -\Delta G^*_{\text{int}} \]  

yielding the result

\[ RT \ln K_{\text{int}} = \Delta G_{\text{el},p} - \Delta G^\circ + \Delta G_{\text{cav}} \]

which is valid for either \( K_t \) or \( K_p \).

The electrostatic cooperativity is determined upon insertion of Equation D.4 into Equation D.6:

\[ \Phi(Z) = ZZ_p \left( A - \frac{B\sqrt{I}}{1 + C\sqrt{I}} \right) \]  

The constants can then be evaluated for aqueous solutions at 25°C to be

\[ A = \frac{7.122}{R_i}, \]  
\[ B = 2.341, \]  
\[ C = 0.3287a, \]

where \( I \) is in mol/l, and \( R_i \) and \( a \) are in Å. The distance of closest approach is determined from the centers of the macromolecule and the ligand.
Determination of $K_{\text{int}}$ can be achieved using Halicioglu and Sinanoglu's (1969) expression for the free energy change of cavity formation upon transferring a solute molecule into solution:

$$\Delta G_{\text{cav}} = F + \Omega \text{RT},$$  \hspace{1cm} (D.13)

where

$$F = [NH + 4.8N^{1/3}(\kappa^e - 1)V^{2/3}] \omega, \text{ and}$$  \hspace{1cm} (D.14)

$$\Omega = [NH + 4.8N^{1/3}(\kappa^e - 1)V^{2/3}]\sigma/\text{RT}.$$  \hspace{1cm} (D.15)

Upon inserting equations D.13 and D.4 into Equation D.7 one obtains the solution

$$RT \ln K_{\text{int}} = F - \Delta G^\circ + \frac{Z_P^2}{2} \left( A - \frac{B\sqrt{I}}{1 + C\sqrt{I}} \right) + (\Omega - \Lambda)\text{RT}.$$  \hspace{1cm} (D.16)

The latter term on the right hand side of the equation contains the terms $\Lambda$ and $\Omega$, which are commonly referred to as the intrinsic salting-in and salting-out coefficients, respectively.